New Approaches for Primary Diagnosis and Screening of Nasopharyngeal Carcinoma by Using Epstein-Barr virus Markers

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CHAPTER 1

General Introduction

1. General features of EBV

1.1. History of EBV-findings

Dr. Dennis Burkitt during his work in Uganda from late 1950s and early 1960s discovered a common tumor in local children later known as “Burkitt's Lymphoma” (BL). His suspicion towards an etiological role of environment and infection remained unanswered (39, 40), until 1964, when Anthony Epstein, Yvonne Barr and Bert Achong succeeded in culturing cells from BL biopsy material in vitro and identified in these cells a novel herpesvirus using electron microscopy (93, 94). This virus was subsequently named as Epstein-Barr virus (EBV) or Human Herpesvirus 4 (HHV4).

By means of serological techniques based on the use of the original BL cell lines, it was found that EBV reactive serum antibodies were detectable (present) in about 90% of the world population, classifying EBV as a ubiquitous human virus. Seroconversion from negative to positive marks primary infection after which the virus persists for life, well balanced by the host's immune system, as reflected by the lifelong presence of low level EBV-infected cells and anti-EBV reactive antibodies and T-cells in the circulation (348). When infection takes place during early childhood, EBV remains largely asymptomatic. Delayed primary infection as mostly happens in western populations may cause a clinical syndrome called infectious mononucleosis (IM), a self-limiting, benign lymphoproliferative disorder. In the recent 20 years EBV has been etiologically related to multiple disease entities, including several types of malignancy (see table 1.). In 1997 WHO has recognized EBV as “class 1 human carcinogenic virus” (177).

1.2. Features of herpesviruses related to EBV

Herpesviruses are a family of DNA viruses commonly found in humans and animals with about 100 different species being identified. The human herpesvirus family comprises of three (3)

Table 1. Expression of EBV latent genes and related disease (adapted from Middeldorp et al. (294)

Latency type	Gene products	Disorders
I	EBERs, EBNA1, BARTs	Burkitt's lymphoma
II	EBERs, EBNA1, BARTs, LMP1, 2A, 2B, EBERs, EBNA1, BARTs, LMP1, 2A, 2B	Nasopharyngeal carcinoma Hodgkin's lymphoma, NHL immunocompetent
III	EBERs, EBNA1, 2, 3a, 3b, 3c, LP, BARTs, LMP1, 2A,	AIDS related lymphoma, Infectious mononucleosis, PTLD
others	EBERs, EBNA1, LMP2, EBNA1, BARTs	Gastric cancer Hepatocellular carcinoma

With BARF1 expressed additionally in NPC and GC

subfamilies, i.e. α-*herpesvirinae* (Herpes simplex virus (HSV) type 1,2, and varicella zoster virus (VZV), β-*herpesvirinae* (cytomegalovirus (CMV), human herpesviruses (HHV) 6,7) and γ-*herpesvirinae*. The γ-*herpesvirinae* are further divided into two (2) genuses, i.e., *Lymphocryptovirus* (Epstein-Barr virus (EBV)) and *Rhadinovirus* (Kaposi's sarcoma-associated herpesvirus (KSVH)) (410).

Herpesviruses share several features: (1) genetically stable large genomes consisting of double-stranded DNA encoding a variety of enzymes involved in nucleic acid metabolism, DNA synthesis, protein processing, and host cell regulation (160, 177), (2) the ability to establish latent infection in their host cells, some inducing proliferation of latently infected cells, (3) similar virion structure, basically consisting of a DNA-wrapped protein core enclosed by a symmetrical capsid, a phosphoprotein tegument layer and a lipid envelope containing glycoproteins (294).

1.3. Structure of EBV

The EBV virion consists of toroid-shaped protein core, containing a linear-double stranded DNA molecule of 172 kb surrounded by an icosahedral capsid, amorphous tegument, and lipid envelope with viral glycoprotein (gp) spikes on its surface (211). The diameter of the viral particle is 120-200 nm, and consists of 25-35 proteins and host specific phospholipids derived from nuclear membrane (194). gp350/220 is the most abundant in the envelope, followed by glycoprotein gH, gB, gM, gp42, gL, gp78, gp150, and gN. The tegument consists of BLRF2, BRRF2, BDLF2 and BKRF4, and host actin, cofilin, tubilin, hsp90 and hsp70 (194, 351, 410). Capsid formation initiates around a scaffold-core (BdRF1) which degraded by a viral protease (BVRF1) upon DNA entry into the matrix capsid. The small capsid protein BFRF3 closes the outer shell of the capsid linking the tegument. Following passage through the nuclear membrane, the tegumented capsid becomes enveloped in the cytoplasmic membrane of the Golgi-region, from which it is cleaved by exocytosis.

1.4. The genome of EBV and open reading frames (ORF)

The EBV genome in the virion consists of a linear double stranded DNA molecule, whereas in host cells EBV DNA persists as a closed circular molecule reliably amplified by the host DNA polymerase machinery upon each cell division. EBV isolates are divided into two strains, type 1 and 2 (or A and B), based on sequence variation on EBNA2 (Epstein-Barr nuclear antigen) and EBNA3 proteins. EBV1 is more prevalent than EBV2 in Western populations; however, EBV2 infection is prevalent in central Africa, in New Guinea, and among Alaskan Eskimos (479). EBV type 1 and 2 differ in the efficiency of growth transformation of the host cell, EBV1 being the most efficient (350). Co-infection with both EBV types is frequently detected in HIV infected patients but can also occasionally occur in normal individuals (450). Between EBV type 1 and 2 the sequence variation for EBNA2A, 3A, 3B, and 3C is 36%, 10%, 12%, and 19%, respectively (211). Sequence variation was also found in LMP1 (418), LMP2 (41), EBNA1 (455), and BZLF1 (326). Other genes appear rather conserved, for example vIL-10 (198), EBER (12, 129, 352), and BARF1 (Hutajulu et al., in prep.).

The EBV genome is approximately 170 Kb in size and contains several internal repeat regions and about 80-100 open reading frames (ORF), only 50-60 of which are characterized in some extent. Some regions are characterized by extensive splicing and some ORF's span the genome terminus, and are therefore only expressed in latent infection (see fig. 1). The ORFs are systematically named according to the size of the BamH1 restriction fragment containing the RNA site start with either rightward or leftward transcriptional orientation (see fig.1.), e.g. BARF1 means BamH1-A, rightward frame 1.

The EBV genetic sequence was resolved as early as 1984 and is assembled based on B95.8 and Raji (EBV type 1) sequences, and annotated as EBV *wt* sequences (101). However, both B95-8 and Raji show some genomic deletions and mutations that are likely derived during extensive in vitro propagation. More recently the complete genome for EBV type 2 (AG876) (87) and the Chinese NPC-linked prototype GD-1 strain were published (486), advancing our knowledge on genome functions in different EBV isolates around the world. The genome is characterized by a short terminal repeat (TR) at both ends, and tandem arrays of internal repeats (IR1-4). Upon infection, linear genome will circularized by joining at the terminal repeats. (see fig.1)

2. Biology of EBV

2.1. Virus entry into host cell

EBV infection occurs at any stage of life, leading to lifelong carriership and reflected by seropositivity. The association of EBV with the development of both lymphomas and carcinomas reflects the dual tropism of EBV for infection of B lymphocytes and epithelial cells, although

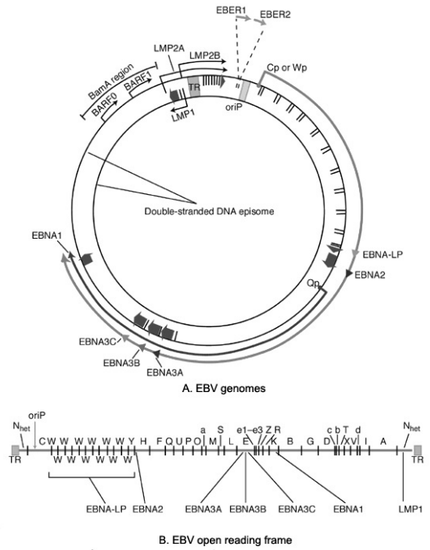

Fig. 1. EBV genome and open reading frame (ORF) showing all latent genes.(Fig adapted from Young and Rickinson (478)

occasionally other cell types can be infected as well, i.e. T-/NK cells (60, 77, 429) and monocytes (367, 444). The infection of EBV in different cell types triggers substantially different activation pathways and is associated with different pathogenic events.

2.2. B-lymphocyte entry

In vivo EBV is mainly transmitted via saliva exchange. Saliva contains EBV virions which are sampled by the tonsil, where infection occurs not at the apical surface of epithelial cells (445), but at the crypt ("invagination") of the tonsil (175). EBV will cross the epithelial barrier, to reach submucosal naïve B cells residing in the mantle zone facing the surface. The oronasopharyngeal lympho-epithelium (Waldeyer's Ring) is believed to be site for EBV primary and persistent EBV infection (175, 333). There are only limited data supporting epithelial cells as primary EBV point of entry. Primary EBV infection may preferentially occur in B lymphocytes, as individuals with B cell deficiency are usually not infected with EBV (102) and the virus is not found, even locally, in oropharyngeal epithelium of these patients. Gratama (132) showed that B cell depletion can even eliminate EBV from the body, showing the important role of B cells in EBV persistence.

The infection of B cell is initiated by attachment of gp350/220 to complement receptor

type 2, named CR2 or CD21 (106, 428). Binding of CD21, triggers host cell activation (69, 428). High density of gp350/220 ensures cross-linking of the CD21 signaling complex, which drives the resting B cell from G0 to G1 phase of the cell cycle. MHC-II is considered an essential co-receptor for EBV entry. Fusion of the virus envelope and host cell membrane is triggered by interaction between HLA class II and virus glycoprotein gp42 which form a complex with glycoprotein gHgL (252, 253), and completed by interaction of that complex with gB (137).

Upon B cell entry, the EBV nucleocapsid is then transported to host nucleus and releases its linear DNA content, triggering gradually the expression of a number of nuclear proteins (EBNAs) and latency-associated membrane proteins (LMPs) (294, 434). Initially, the Wp promoter becomes activated via cellular transcription factors triggered by CD21 activation, leading to EBNA2 and LP expression. EBNA2 is a transcription factor stimulating expression of all nine latent genes. Subsequent methylation of Wp CpG islands (250, 430) leads to switching from Wp to Cp promoter providing more consistent transcription. EBNA2 also drives the expression of LMP1 shortly after infection, leading to establishment of the growth program (latency type III). Expression of EBNA3A and B will downregulate EBNA2 through competition for the cellular factor Rbp-Jk. EBNA3C will activate G1 progression within cell cycle. This will activate B cells to become growth-activated B cell blast not requiring further external signals. EBNA1 is co-expressed via Cp-transcription and provides essential signals for EBV genome maintenance in dividing cells. Activated B cell will migrate to the follicle, and subsequently downregulate Cp-driven EBNA2 and 3A-C, and switching to Qp-driven EBNA1, and EBNA2 independent expression of LMP1 and LMP2A gene expression, known as default program (latency type II) (434). During this process, LMP1 and 2A work in coordination ((43, 45, 143, 331), mimicking T cell help and antigen-specific B cell receptor triggering, to ensure the infected B cell to undergo germinal center reaction, proliferation, and become memory B cells without external signals (434). In the circulation, EBV-infected memory B cell will have all latent genes silenced by promoter methylation, except Qp-driven EBNA1 and the non-coding transcripts EBER1,2 and BARTs. Thus, the viral genome will be maintained within normal memory B cells (162), known as latency program (latency I, EBNA1 only). *In vitro*, immortalized lymphoblastoid cell lines, produced by EBV infection of B-cells, show similar cell surface phenotype (355, 437, 438), cell morphology (316), and latent gene expression profile as seen during natural primary B cell infection (435). Memory B cells are not dividing in culture, but will quickly undergo growth activation in absence of T cell control and can turn into dividing B cell blast. This also illustrates the importance of T-cell control in preventing overwhelming B-blast outgrowth *in vivo*.

2.3. Epithelial cell entry

The mucosal epithelium of the oropharyngeal surface may serve as a portal of entry for EBV during primary infection and as the pathway of exit for progeny virions during systemic infection (445). EBV replication can rarely be detected in post-mortem tongue biopsies of immunocompetent virus carriers (115). Productive EBV infection of the oral (tongue) mucosal epithelium has been shown in AIDS related oral hairy-leukoplakia (OHL) a hyperplasia combining latent and lytic EBV gene expression (133, 156). In gastric adenocarcinoma (GC) and nasopharyngeal carcinoma (NPC), latent EBV gene-expression is seen in the epithelial tumor cells, resembling the default program, with variable (NPC) to negative (GC) LMP1 expression. Recently, ex-vivo explant culture of tonsillar epithelial cells confirmed that infection of oropharyngeal epithelium is part of natural EBV-host interaction (333). These data reflect *in vivo* access of EBV to mucosal epithelial cells.

The mechanism of infection, virion release, and virus spread in epithelial cells is not well understood. Several models have been proposed to explain how EBV gains access to epithelial cells. Sixbey and Yao (392) suggested that EBV is produced by submucosal B cells and binds to submucosal EBV-specific dimeric IgA to enter basal oral epithelial cells by endocytosis via polymeric Ig receptor. CD21 and the MHC-II co-receptor is normally not found in epithelium (296), however during episodes of local inflammation/ infection IFN-γ might induce HLA class II and CD21 expression in the epithelial cells (164, 461). Recent studies suggest a role of other cell types such as Langerhans cells (LC) (452) and monocytes (444) as transporter of EBV from EBV infected resting memory B cell in peripheral blood into oral epithelial cells.

Tugizov et al. (445) studied EBV infection in polarized tongue and pharyngeal epithelial cells, and suggested three CD21-independent pathways, i.e. (1) by direct cell-to-cell contact of apical cell membranes with EBV infected B lymphocytes, (2) by entry of cell-free virions through basolateral membranes, mediated in part through interaction between β1 or α5/β1 integrins and EBV-BMRF2 envelope protein containing an RGD motif, and (3) by virus spreading laterally to adjacent epithelial cells via desmosomes and cell juctions. *In vitro*, the second model is most favourable (179, 378, 445). Virions can egress epithelial cells both via apical and basolateral membrane, providing bi-directional spread to saliva and B-cell compartments. Recently, Shannon-Lowe et al. (378) and Pegtel et al. (333) suggested direct transfer of B cell associated EBV to epithelial cells via cell to cell contact.

Virus attachment and penetration to epithelial cells is mediated by dimeric complex of gHgL wich binds to yet unknown receptor (gHgLR), suggested to have conformational changes to provide virus attachment and penetration to epithelial cells (444). In vitro, gHgL null virus failed to bind to gastric carcinoma cell line (AGS) (297, 318).

Gp42 that plays important role in B cell binding via MHC class II, does not have important function in EBV epithelial entry, as epithelial cells normally do not express HLA class II molecule. (32, 454). gp42 can function as tropism switch from B lymphocyte to epithelial cells as described in detail by Borza and Hutt-Fletcher (32). The study compared EBV infected B lymphocyte and epithelial cells (32), and revealed that HLA-class II negative epithelium derived virus contains more gp42 compared to HLA class II positive B cell-derived EBV, and virus prepared in HLA-class II negative epithelial cells was found to be up to 100-fold more infectious for B lymphocytes than the same amount that produced by HLA-class II positive B lymphocyte, and *vice versa*. Virus derived from B cell was 5 fold more infectious to epithelial cells. This reflects the fact that in a HLA-class II positive virus-producing B cells, gp42 becomes target for HLA class II degradation pathway, which may reduce the concentration of gp42, compared to HLA-class II negative virus-producing epithelial cells.The loss of trimolecular complex of gH-gL-gp42 complex reduces the possibility to for HLA-class II dependent entry. Thus virus produced in MHC-II expressing B-cells, has higher tropism from epithelial cells and *vise versa*. Viral infection of epithelial cells enhances virus production and may ensure the ability of the virus to move back to B-cell compartment.

2.4. Latent EBV replication

In vitro, EBV infected memory B cells are able to divide indefinitely and the EBV genome is maintained as episome, replicating once per cell cycle simultaneously with the host chromosomes (243). Replication and maintenance of EBV episome is regulated by *oriP* in *cis* and EBNA1 protein in *trans*. *oriP* ensures the EBV plasmid to replicate (275, 343, 474) and segregate (217) equally to each daughter cell. EBNA1 binds to *oriP* as multimeric dimers (see below) and links the EBV-genome to host chromosomes via so-called AT-hooks (372). EBNA1 lacks enzymatic

Table 1. Expression of EBV latent genes and related disease (adapted from Middeldorp et al. (294)

Gene products	Function
EBNA 1	Maintenance of EBV episome through sequence-specific binding at OriP and chromosomes (218) Transcription factor for EBNAs and LMP1 (294, 434). Destabilises p53 via interaction with USP7 (366) Stable EBV protein preventing proteosomal breakdown via gly-ala repeat (248, 476).
EBNA 2	EBV transformation together with EBNA-LP Interact with RBP-Jκ to transcriptionally activate CD23 and other cellular genes and viral LMP1 and LMP2A (9).
EBNA-LP	Upregulation of transcription factors needed for B-cell growth (286)
EBNA3 family	transcription regulators (5, 258) 3A: interact with EBNA2 effect on RBP-Jκ binds to CtBP (9) 3B: transcription regulator (9) 3C: disrupt cell cycle checkpoints (9)
LMP1	Essential for EBV transformation of B cells in vitro, and drives proliferation (76, 215) through NF-êB, AP-1 and JAK/STAT activation (83, 126, 301, 363) Mimics CD40 activity by providing growth and differentiation signals to B cell (98, 331) Upregulation of anti-apoptotic proteins (Bcl-2, A20) (117, 145) Inhibits p-53 mediated apoptosis (118) Induces malignant transformation (453) Induces malignant progression by inducing pro-angigenesis factors as VEGF, bFGF together with Cox-2 and MMP-9 (in epithelial cells) (166, 167, 304, 449, 477)
LMP2A	Inhibits signaling through BCR and promotes survival during B cell development (337)
LMP2B	Modulates LMP2A (337)
EBER1,2	Inhibit apoptosis by with IFN-α through binding PKR (222) Induce IL-10 expression (219)
BARF1	Induces malignant transformation in primary epithelial cells (459) Provides apoptosis-resistance by activating bcl-2 (381) Modulates monocyte activation (65, 411)

functions; therefore EBV latent replication solely relies on cellular DNA polymerase activities (4, 116, 176, 295). *oriP* contains two cis acting elements, i.e. the family of repeats (FR) and the dyad symmetry (DS) element, with 16 and 4 EBNA1 binding sites, respectively (342, 343). DNA synthesis starts at or near DS (225) with replication forks either pausing or terminating at FR (121). The interaction of EBNA1 and FR supports long term reliable EBV plasmid replication in dividing cells (225, 284) as well as enhance transcription of other latent genes in *cis* (122, 415) .

At the initiation of replication, homodimers of EBNA1 binds EBV genome via the carboxy terminus (amino acid 451-640) to FR and DS of *oriP* (276). By means of cellular enzymatic activities *oriP* plasmids replicate semiconservatively during S phase (1, 4). The EBNA1 amino-terminus links host mitotic chromosome through DNA binding/ dimerisation domain at A (amino acid 33-89) and B (amino acid 328-378) domain, and by heterotypic interaction with cellular proteins, i.e. p32/ TAP to A and B domain (57, 456), and EBP2 to B domain (388). Other cellular factors are implicated to be recruited by EBNA1 to the origin site, such Rch/ importin α (107, 216), origin recognition complex (ORC) (84, 369), MCM helicase complex (54), replication protein A (490), and telomere repeat binding factors 2 (TRF2) (82). During metaphase, the EBV plasmids tethers symmetrically to sister chromatids (199, 276, 372) and exploit the centromere for plasmid segregation. These events

ensure a stable EBV episomal maintenance during host cell divisions. Besides its role in viral episome maintenance EBNA1 serves as transcriptional enhancer and destabilizes p53 via interference with the USP7-HAUSP/MDM2 de-ubiquitination complex, potentially contributing to oncogenesis, although EBNA1 itself is not a direct oncogene (200, 366, 489).

2.5. EBV reactivation

To persist for a lifelong period after primary infection, EBV hides latently in memory B cells and has developed subtle routes for transmitting its genome to new host cells. The latent state of EBV is periodically changed to lytic stage (i.e. reactivation), which leads to amplification of viral DNA, release of infectious virions, and host cell death (211, 212). Reactivation is related to memory B cell differentiation into plasma cells (436). However, depending on the cell type and inducing signals, EBV reactivation can also be partial, leading to expression of a limited number of lytic phase genes, without new virus production (i.e. abortive activation).

In vivo, environmental changes such as chemical influences (butyrates, nitrosamine, stress hormones) (138, 190, 371, 375), gamma irradiation, inflammation and over-expression of p53 (251) are suspected to trigger EBV to activate the lytic cycle. In vitro, in latent EBV-infected B cells a variety of treatments such tetradecanoyl phorbol acetate (TPA), Ca^{2+} ionophore, sodium butyrate, anti-IgM, transforming growth factor beta 1 (TGF-β1), and hypoxia (97, 123, 192, 254, 344) can mimic this effect.

During lytic infection, similar to other Herpesviruses, viral genome activation is divided into three coordinately regulated, sequentially ordered phases of gene expression designated as (1) **immediate-early (IE),** comprising viral transcription factors, essential for the lytic switch (2) **early (E),** comprising enzymes manipulating host cell metabolism and nucleotide synthesis prior to actual viral DNA replication, and (3) **late (L)**, includes virion structural and non-structural proteins. Late protein synthesis is dependent on new viral genomes and can be blocked by inhibition of viral DNA polymerase (142, 171, 211). Lytic replication includes cascade of gene activation to express full structure of EBV virion. The switch to the lytic cycle is mainly controlled by two of the IE gene

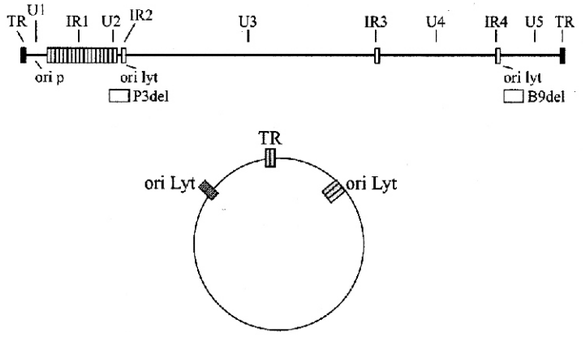

Fig.2. EBV genome characterized by short terminal repeat (TR) at both ends, and tandem array of internal repeats (IR). Deletion of genome sequence in P3HR-1 (P3*del*) and B.95.8 (B9*del*) EBV cell lines also shown. EBV clonality showed by its identical TR length. Upon infection, EBV enters host cell as linear DNA, and forms episome in the host' nucleus.
Lytic replication started from one of two origin of replication (*oriLyt*) and proceed repeatedly around the circular viral episome, producing long linear concatamers of viral genome. (adapted from (182, 212)

products, BZLF1 and BRLF1, which functions as a transcriptional activator to initiate an ordered cascade of viral lytic gene expression (212)

The Akata cell line (derived from Burkitt's lymphoma; latency type I) is the most frequently used model to study EBV reactivation *in vitro* (426). In the absence of LMP protein, EBV can be reactivated by mimicking antigen binding to BCR (B cell receptor), via cross-linking the surface Ig with anti-Ig antibody (386, 423). BCR crosslinking activates P13K (phosphatidylinositol 3-kinase) and via induced host cell transcription factors (Lyn, Syk activation and YY1 (298, 299), SP1, SP3 (262, 341), C/EBP-alpha (421), SMAD, EGR1, CREB (110), ATF-1 and ATF-2/c-jun heterodimer (2, 261, 457), induce transcription of Zp and Rp, leading to expression of immediate early genes BZLF1 and BRLF1 encoding protein Z (ZEBRA, Zta, EB1) and R (Rta), respectively (2, 36, 75, 185, 223). Together these proteins are required to initiate the cascade of lytic gene expression. Zta and Rta will function as transcription regulators and induce the expression of early genes involved in EBV lytic DNA replication (BMRF1, BALF2 et al., see below), as well as their own genes (62, 163), RNA transport and stability factors (BMLF1/SM) (67), anti-apoptosis factors (BHRF1 and BALF1 (22)), homologues of host bcl-2 and immune evasion genes (i.e. BGLF5 , (354, 493), BNLF2a (159), and BARF1 (as soluble receptor of CSF-1)) (411, 431).

For lytic replication, the viral *oriLyt* (origin of replication) is required in *cis* and viral core replication proteins in *trans* (108, 109). The core replication proteins include viral DNA polymerase (BALF5), DNA polymerase processivity factor (BMRF1), helicase (BBLF4), primase (BSLF1), primase associated protein (BBLF2/3), and single stranded DNA binding protein (BALF2) (109, 255). Other early proteins function as enzymes such as thymidine kinase (TK; BXLF1) (260), viral dUTPase (112) and ribonucleotide reductase subunits (BORF2 and BaRF1) together, ensuring sufficient nucleotide substrate in non-replicating cells.

Late viral proteins, requiring viral DNA synthesis as prerequisite for their expression, include proteins for virion assembly, including BNRF1, BFRF2/3, BLRF1/2/3 and BcLF1 (major capsid protein), virion envelopment i.e.BLLF1 (encoding gp 350/220), BXLF2 (encoding gp85), BZLF2 (encoding gp42), BKRF2 (encoding gp25), and also immunomodulation factors such as BCLF1 (vIL-10) (summarized in (182)) Mature EBV virions will be released from host cells in yet an unknown mechanism. Viral glycoproteins induce immune responses, and are used as target in vaccination and immunotherapy against EBV infection.

Importantly, viral DNA synthesis and consequently virion production can be blocked by treating “activated” EBV infected cells with chemical agents directly interfering with EBV BALF5 encoded DNA polymerase (eg. Phosphonoformic acid also called Foscarnet) or nucleoside analogues like (val)gancyclovir and (val)acyclovir (known as Vancyte and Zovirax), requiring specific phosphorylation by virus encoded TK. These agents are therefore widely used as specific antiviral agents effective in diseases linked to virus replication, e.g. OHL. These agents have no effect on latent EBV DNA replication which proceeds via an alternative mechanism involving the host polymerase complex as discussed before.

3. Non-coding transcripts

Of all latency types of EBV infection, two products are always expressed, i.e. EBV-encoded small nonpolyadenylated RNAs (EBER1 and EBER2) and BamH1-rightwards transcripts (BARTs) (see table 1). Transcript of EBERs and BARTs are only found in RNA format, with yet unclear functions. The persistent and abundant expression, suggest that EBER and BARTs play an important biological role. Recently, 3 families of EBV micro-RNA (miRNA) were identified, encoded within the BHRF1 and BART transcripts, which are still at an early stage of exploration (42, 334)

3.1. EBER1 and EBER2

EBV-encoded small RNAs (EBERs) are the most abundant viral transcript in latently infected cells (357). EBERs are encoded by 1000 bp EcoRI J fragment of EBV genome transcribed by RNA polymerase III. These genes are highly expressed, upto 1-10 million copies per cell, and comprise of uncapped, two untranslated and nonpolyadenylated RNA transcripts of 167 (EBER1) and 172 (EBER2) nucleotides long, separated by 161 bp. EBERs form a stem loop structure by intramolecular base-pairing, giving rise to double-stranded RNA (dsRNA)-like molecule. For its relatively abundant expression, EBER (especially EBER1, as it showed higher stability compared to EBER2) has been extensively used as golden standard for detecting EBV latent infection in tissue specimens (53). However, Yao et al. (473) showed a variable EBER expression --from negative to abundant -- even in one tissue specimens, suggesting a possibility of EBER negative status of EBV latently positive cells (425).

EBERs are predominantly localized in nucleus but are also found in the cytoplasm where they form complexes with cellular proteins, i.e. dsRNA dependent protein kinase (PKR) (222, 356, 470), ribosomal protein L22 (92, 440), La antigen (244), and retinoic acid-inducible gene RIG-I (361, 362). The association with cellular protein indicates EBER's biological role. EBERs induce resistance to IFN-α induced apoptosis by direct binding with PKR, inhibiting its phosphorylation (311, 463), and modulate its downstream anti- and pro-apoptotic proteins (463). EBERs can also induce expression of cellular growth factors, such as IL-10 in B cell (219), and IL-9 in T cells (472), and Insulin-like growth factor1 (IGF-1) in epithelial cells (183, 184). The productions of cytokines are found to be modulated through RIG-I signaling activation by EBER (362).

Recently, Yajima et al. (469) shows that EBER contributes to efficient growth transformation of B cells. Still, EBERs are considered ”non-essential” for B lymphocyte transformation, apoptosis resistance and cytokine production, as these effects may be “masked” by more powerful LMP-1 functions (469).

3.2. BamHI-A rightward transcripts (BARTs)

The BamH1-rightwards transcripts (BARTs) also known as complementary strand transcripts (CST), are a group of noncoding polyadenylated RNAs, and was originally identified and abundantly expressed in NPC tissue (161). BARTs are also detected at low level in BL tissue, B-LCL (35, 55, 134, 201, 340), EBV-GC tissue (416), HD (491), and in normal EBV persistence in the peripheral B-cell (56, 125, 213). Although deletion of BARTs region from EBV genome does not impair ability of EBV to immortalize B cells in vitro, the consistent detection of the BARTs in EBV-assocated tumors implies that they may have some-as yet unidentified- contribution in vivo.

Splicing of BART-RNAs is complex (358), with at least 16 different-partly overlapping exons identified in cDNA (395). The expression of whole BART gene has not been fully understood, but several ORFs have been identified, including RPMS1, A73, and BARF0 and its further spliced to RK-BARF0 (119, 213, 395). Biological properties of these ORFs were characterized only through exogenously-expressed proteins (119, 228, 395, 491). RPMS1 located in exon 4 (and exon 5 in some CST), is reported to interact with CBF1, the nuclear effector of Notch signaling pathway and a promoter targeting EBNA2. A73 is formed by component of exon 6 and 7 expressed in cytoplasm of transfected cells, and reported to interact with cellular protein RACK1, an adaptor protein in regulating signaling from protein kinase C and Src tyrosine kinase. RK-BARF0 located in exon 7, was reported to interact with extracellular ligand binding of Notch4 and translocates part of Notch4 to the nucleus and can turn on LMP1 expression in BL cells. However, above experiments used *in vitro* transfection with artificial RK-BARF0 containing constructs. Using specific monoclonal antibodies actual expression of a RK-BARF0 protein in EBV-immortalised cells was never detected

despite in situ detection of BART RNA in the same cells and antibody responses to the putative RK-BARF0 protein were not found *in vivo* (18). Similar findings were reported for A73 and RPMS1 gene products, which has led to the assumption that BART expression serves another purpose in the EBV-infected cell (18, 439). Recently, it has been found that BART intronic region can generate BART-miRNA including two different miRNA clusters (see below).

3.3. EBV microRNAs (miRNAs)

miRNAs are small single stranded RNA molecules, encoded by the genome that are not translated into proteins; rather, they control the expression of genes. Regions of the genome that encode miRNAs are transcribed in the cell nucleus. Nascent miRNA transcripts are initially processed into long (up to several kilobases in length) precursor miRNAs that are then transported into cytoplasma, and sequentially cleaved by two enzymes, Drosha and Dicer, into small functional RNAs (~22 nucleotides). These miRNAs are subsequently incorporated into an RNA-induced silencing complex (RISC), which suppresses the translation and/or promotes the degradation of target messenger RNAs (mRNAs) by binding to their 3'-untranslated regions (3'-UTRs). Functions of miRNA are either repression of translation, inducing mRNA degradation or deadenylation of their target RNA molecules (336).

EBV is the first human virus reported to encode miRNAs (334). EBV encodes at least 23 miRNAs, divided into three clusters, BHRF1 (3 miRNAs), and two clusters of multispliced BART, 12 in cluster one, and 15 in cluster two, while miR-BART2 is an individual miRNA (42, 135). The B95.8-strain of EBV which has a deletion of 12 kb but is fully transformation competent encodes only eight miRNA genes [miR-BHRF1-1-, -2, -3, miR-BART-1, -2, -3, -4 and the partially deleted miR-BART5 (42). The targets of miRNAs are not completely understood. miR-BART2 suggested to play a role in cleavage of virus DNA polymerase (BALF5), as miR-BART2 is antisense to 3'-UTR of BALF5 mRNA. (16). Xing and Kieff (467) have detected high level of miR-BHRF1-1,-2, and 3 as a characteristic of EBV latency type III infection encoded within introns of Cp-driven EBNA transcripts (42, 467). Although the target is still unknown, it is predicted that miR-BHRFs play a role in latency type III infection. miR-BART cluster 1 and 2 found to be expressed in EBV-infected epithelial cell lines (467). Lo et al. (266) showed that miR-BART cluster 1 targets LMP1 3'UTR and negatively modulate LMP1 expression. Overexpression of LMP1 can inhibit EBV induced cell growth and potentiate apoptotic effects induced by stress of chemotherapeutic treatment, such as cisplatin (264, 274). Co-transfection of miR-BART cluster 1 with CNE-EBV cell line supressed LMP1 protein expression hence reduced cisplatin-sensitivity as well as dose-dependent modulation of NF-κB activity, thus potentiating cell survival (266). The full understanding of the role of EBV encoded mRNAs in EBV biology and malignant outgrowth requires further studies.

4. Immune responses to EBV and immune evasion

EBV is a human virus that lives within cells of the immune system and has evolved a series of adaptations to limit elimination and ensuring a lifelong persistence. Most population worlwide are infected by EBV, without apparent symptoms or disease suggesting effective control by the host. To counteract the strong transforming capacity of EBV, the host has developed potent mechanism of immune control (see fig. 3.). At times of immunodeficiency at some point EBV can take over and lead to virus associated diseases. In most cases these diseases are associated with replication of EBV-transformed cells rather than virion production. Virion production is mainly detected in saliva. In fact, the EBV-host interaction is a lifelong dynamic balance between virus replication, proliferation of virus-infected cells and host's surveillance.

4.1. Innate immune response

The tonsil is the main entry and homing site for EBV, thus being an important organ for first line immune defenses. Innate immunity is driven by molecular and cellular mechanisms, the former including interferon -α, -β, cytokines, and the latter including dendritic cells (DC), and natural killer (NK) cells. Since IM patiens seek medical help only after clinical manifestation occurs, the very early stage of EBV infections has not been investigated *in vivo*. In vitro, EBV-infected peripheral-blood mononuclear cells (PBMC) showed production of interferon (IFN)-α mainly by B and NK cells (214, 273), reaching a peak after 24 hour. In EBV infected B cells, EBERs and EBNA2 and-LP mediate resistance to IFN-α (7), and LMP1 seems to protect against apoptosis by IFN-α (145). Therefore, IFN-α induced effects have limited ability to protect the host cell from EBV infection. *In vitro*, tonsillar NK cells are able to limit B cell transformation through the release of IFN-γ in the presence of dendritic cell producing IL-12. (160). However, in immunosuppressed patients NK activity does not provide sufficient immune protection (317). DC -- via still unknown activation stimuli -- play a role in immune reactivity towards EBV, by cross-presentation of EBV antigens to T cells in different stages of maturation (27, 390, 413) and also activate NK cells (105). Innate immune response is important at early stage of infection, and provides necessary signals for induction of the adaptive (antigen specific) immune response.

4.2 Adaptive responses

Adaptive immune responses are mediated by antigen specific B- and T-lymphocytes in different subclasses, such as CD8+ cytotoxic T cells, CD4+ T helper cells and regulatory cells.

4. 2. 1. $CD8^+$ T cell response

$CD8^+$ T cell cytotoxic T cells (CTL) play a major immune defense against tumor cells and endogenous infection, including virus. Endogenous (viral) proteins are processed and cleaved by proteasomes, and resulting peptides are translocated into lumen of ER by transporter-associated with antigen processing (TAP1,2), and loaded onto newly synthesized MHC class I molecules. MHC class I molecules presents the peptide to $CD8^+$ T cells which activates the EBV-peptide specific pool of $CD8^+$ T cells. The responding population can be quantified by various activation markers, like IFN-γ production using FACS or ELISPOT (289). $CD8^+$ T cells contain perforin and Fas-FasL activity for direct killing of tumor cells as well as virus-infected cells. Still, target cells may develop immune evasion mechanisms to escape elimination (see “immune evasion”).

$CD8^+$ T cells produce ample protection to limit proliferation of EBV-infected cells *in vivo* (221, 289, 349). $CD8^+$ T cell to latent and lytic EBV expand rapidly upon primary infection and cease rapidly as well, leaving barely detectable EBV infected-memory B cell (see “B cell entry”) maintaining a persistent latent infection. Periodic reactivation produces new EBV-infected cells, which may expand the pool of memory EBV $CD8^+$ T cells. (fig. 4).

In acute disease, such as IM shows extremely strong oligoclonal activated ($CD69^+$/$CD38^+$) $CD8^+$ T cells which are mostly perforin/granzyme B positive (397). The response are directed towards lytic (IE and E) rather than EBV latent proteins EBNA3A,B,C (400). Without further stimulus, these activated T-cells will die rapidly by apoptosis, because of their low level of anti-apoptotic bcl-2 and Bcl-x, and pro-apototic Bax (44, 427). Subdominant reactivities to other EBV antigens will appear several months after infection, showing difference specificities between primary and memory $CD8^+$ T cell pool (160).

Asymptomatic EBV carriers were shown to have quiescent $CD8^+$ memory T cells

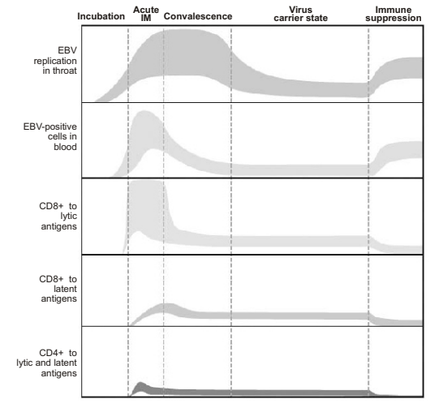

Fig.3. dynamics of adaptive immune components versus EBV infection during primary infection, latent state, and reactivation of EBV. Immune system rapidly eliminates EBV upon primary infection. It may reduce but not eliminates the number of EBV-infected cells. Host-virus balance is created during the virus carriership, and EBV can overpower the immune system in the immune suppressed individuals (adapted from (160, 348)

($CD69^+$/$CD38^-$) that are perforin/granzyme and Bcl-2 negative. $CD8^+$ memory T cells circulate to control the asymptomatic state (28, 91, 99, 158). Immunodominant epitopes are derived from the EBNA3A, B, and C family (30, 131, 208, 306, 382, 399), and subdominant epitopes derive from LMP2, EBNA1, with even less responses to LMP1 epitopes (see fig. 5) (159, 259, 289, 291, 409). Recently, Immunodominant epitopes of Chinese EBV-NPC latent antigens have identified (259). The same study also showed that no immune-impairment exists in NPC subjects compared to healthy virus carrying individuals from the same region.

EBNA1 is protected by its glycine-alanine repeat (GAr) domain from antigen processing (247). Low concentrations of EBNA1-specific $CD8^+$ T cells may be generated from proteosomal breakdown of recently synthesize defective ribosomal products (DRiPs) (303, 432). Compared to full length EBNA1, a recombinant EBNA1 deleted of GAr induces 4x higher IFN-γ responses by CD8+ T cells (240). In BL cells, the generation of $CD8^+$ T cell to EBNA1 can not be targeted because of low expression of TAP and immunoproteasome causing impairment of MHC class I presentation (154, 209, 354).

In the lytic stage, $CD8^+$ T cells are mostly directed to (immediate) early proteins and show markedly decrease from IE, to E with the poorest in L (reviewed in (160)) (see fig. 5). During IE stage, the expression of cell surface molecules of MHC class I are 4-5 times decreased relatively to latent phase infection (203), as well as impairment of TAP antigen transporter (345). EBV lytic phase is important for periodic expansion and virus transmission, therefore they have to avoid immune surveillance. Recently it was revealed that the Early protein DNAse (BGLF5) specifically degrades mRNA encoding MHC-I molecules, thus interfering with antigen presentation.

The proportion of $CD8^+$ T cell specific for EBV epitopes seems to be stable over time, however a rise of $CD8^+$ T cells was observed in individuals over 60 years old (68, 325).

4. 2. 2. $CD4^+$ T cell response

In comparison to the large number of EBV-reactive $CD8^+$ T cells in the acute phase of

infection, $CD4^+$ T cells are present at lower numbers and with less clonal diversity to EBV antigens. However, recent findings emphasize the importance of $CD4^+$ T cell effector functions as helper T cell to maintain functional $CD8^+$ T cell and production of antibody, and independently eliminate/ restrict EBV-infected cells expansion.

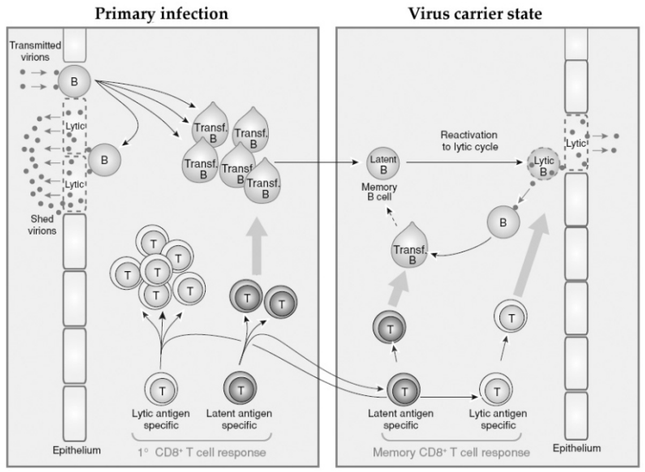

Fig.4. Diagrammatic representation of virus-cell interactions and $CD8^+$ T cell responses in primary EBV infection (IM) and in the memory pool of healthy EBV carrier. Primarily, EBV infects B cells and may spread as cell free virus (virion) to epithelial and B cells. CD8+ T cells generated to an array of latent and lytic EBV antigens, and causes polyclonal $CD8^+$ T cell expansion at primary infection, and resolve rapidly to specific $CD8^+$ T cell in the memory pool to eliminate EBV infected B cells, which may then expanded upon antigen recognition during EBV re-infection of B cells or entering lytic stage (fig adapted from Hislop (160)

In acute infection, $CD4^+$ T cell numbers drop gradually, and reach a steady state at persistent infection (10). $CD4^+$ T cells in primary infection are directed to individual lytic (BZLF1 higher than BMLF1) and latent (EBNA3A) (338, 466) antigens, with less reactivity to EBNA1 (338). During early event of lytic phase, MHC class II surface molecules decrease twofold compared to the latent phase, limiting $CD4^+$ T-cell antigen recognition (203). $CD4^+$ T cells also recognize L antigens such as BCRF1, gp350, and gp110, stimulated by cross-presentation via DC, because virus structural proteins are released from infected cells (159) .

Memory $CD4^+$ T cell, mostly directed towards latent antigens, with EBNA1 (half of C-terminus) being dominant, also recognize subdominantly to EBNA2, EBNA3 family, and lesser to LMP1 and LMP2 (N terminus-hydrophobic part) (242). Intracellular antigen can be generated into a peptide by cross presentation of fragments of virus-infected cells taken up by DC (27), autophagy (330, 370), or by tumor cells triggered to present EBV antigen to $CD4^+$ T cell (reviewed in (303)). Parallel to identification of epitopes for $CD8^+$ T cells in NPC patients, it was found that $CD4^+$ T cells predominantly target EBNA1 compared to LMP1 and LMP2. The concentration of $CD4^+$ T cells was shown to correlate with tumor stage (242, 259).

In BL cell lines, where impairment of MHC class I is found, EBNA1 specific $CD4^+$ T cells

were shown to exert immune protection, as “killer”-$CD4^+$ T. (315). $CD4^+$ T cytolytic cells also recognize EBNA2, EBNA3C (272), LMP2a (412), BHRF1 (233), BALF4, and BLLF1 (3) ($CD4^+$ T cytotoxic cells is reviewed in (144)). The killing actions included apoptosis induction by the ligand of death receptor Fas (FAsL) and perforin activity (412). Evidently, $CD4^+$ T cells also able to prevent B cell transformation in vitro (315, 320).

4. 2. 3. $Cd4^+$ T regulatory cell

T regulatory cells (Tregs) ($Boxp3^+CD4^+CD25^+$) constitute 5-15% of $CD4^+$ T cells. First identified by North et al. (14, 24), also found in peripheral blood and several types of tumor cells (265, 322, 368, 462, 465). These cells suppress activation and proliferation of $CD4^+$ and $CD8^+$ (335, 465), and control autoreactive T cells. In EBV-related malignancy tissues such as NPC (236) and HD (282), infiltrating lymphocytes are observed, but immune response found to be restricted. Many of the infiltrating-lymphocytes were later defined as Tregs. $Cd4^+$ helper and Tregs recognize the same EBNA1 (448) and LMP1 epitopes (283), but play opposing roles in immune modulation. (see ”immune evasion”).

4.3. Humoral response

In response to antigen, and with T cells help, B lymphocytes will produce an array of antibody producing plasma cells and memory cells. The latter, will differentiate to plasma cells producing antibody upon renewed contact with antigen. Neutralizing antibody binds pathogens and prevent them from entering cells, while antibody dependent cytotoxic cells (ADCC) interact with surface-bound antibody Fc via Fc receptors on NK for a direct killing (29, 64). Specific antibody responses to EBV proteins in body fluids (serum or saliva) have become a powerful tool for diagnosis of EBV-related diseases as their presence and dynamics correlate with viral biologic activity and antigen exposure to the immune system.

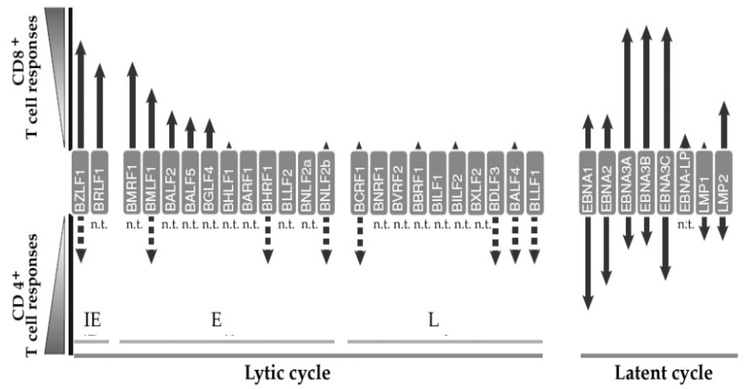

Fig.5. Diagrammatic representation of relatively immunodominance of $CD4^+$ and $CD8^+$ T cell responses to EBV lytic (IE: immediate early; E: early; L; late) and latent antigens in healthy EBV carrier. Dotted line showed $CD4^+$ T cell response was observed but the immunodominance is not yet determined. (n.t.: not tested). (adapted from (160)

During acute infection (IM), IgM, IgA, and IgG response are directed to IE, E and VCA proteins (26). IgA will disappear after one month, whereas IgM will maintain until 3 months (26). IgM to VCA subsequently disappear during convalescence, while IgG to VCA rises until its peak then falls onto steady state levels which persist throughout life. IgG to EA declines faster than IgG to VCA and becomes undetectable or reaches a low level of steady state (148). In acute phase, IgG to EBNA2 is also temporary detected (150), whereas antibody to EBNA1 and gp350/220 reaches peak levels later, at convalescence of IM, and IgG to EBNA1 and VCA persist for life (26, 152). In asymptomatic EBV carriers, IgG anti EBNA1, anti gp-350, anti-BZLF1, anti-BFRF3 and BdRF1 were observed as dominant antibodies specific (348). Antibody to gp350 has been widely studied, since it may prevent the host cell from virus infection with either via Fc-receptor mediated ADCC or virus neutralization action. Development of a vaccine to generate antibody specific to gp350 has been studied for prophylactic purpose (196, 210, 396). In the other hand, IgA to gp350 is associated with NPC development (146) as it may facilitate EBV infection to epithelial cell (392). EBV primarily infects from the mucosal lining, and re-activation may shed virion to the mucosa as well. Specialized antigen sampling M cells in the epithelial are microbial port to access the submucosa where microbial antigens are processed to develop specific IgA antibody responses, including neutralizing antibody. Specific chemokine and adhesion molecules may drive mucosal B cell maturation producing secretory rather than plasma IgA (reviewed in (277). IgA responses to EBV antigens are found in saliva (128, 365) as well as blood plasma (309) giving a diagnostic means for mucosal associated EBV-related disease such NPC.

Interestingly, whereas antibodies to lytic antigens and EBNA1 are readily detected, the humoral response to EBV encoded tumor-associated proteins LMP1,2 (290, 291) and BARF1 (17), Paramita et al. prep.) is marginal and in most individuals not detected at all, even in tumor patients having LMP1, LMP2 and BARF1-positive tumors.

4.4. EBV immune evasion

To persist and exist in the host cell, in the face of a functional immune system, EBV has developed various strategies to evade this potent antiviral CTL response in the immune-competent host. These escape mechanisms include limited gene expression during latent infection, virus replication in immune-privileged tissues, as well as down-regulation of MHC and adhesion molecules (442). The most efficient method for EBV to evade immune-surveilance is downregulate viral genes expression by means of promoter methylation of latent genes in cells constituting latent reservoir (reviewed in (430, 435)), thus maintaining low number of EBV proteins per cell below threshold of immune surveillance.

EBNA1 and LMP1 are two of several proteins expressed in latently infected cells. EBNA1 uses GAr to prevent antigen recognition by proteosome, thus inhibit MHC class I presentation, avoiding $CD8^+$ T cell recognition (248). It is also reported that GAr controlls the expression of EBNA1 by inhibits mRNA translation in *cis* (476), thus limit the EBNA1 expression. Purified and peptide-derived LMP1 induces PBMC (from EBV seropositive individual) causes Treg expansion producing high level of IL-10, which in turn inhibits T cell proliferation and IFN-γ secretion for the survival of infected B cells (283, 329). In addition, LMP1 can actively silence activated T-cells by a conserved sequence in its first transmembrane domain (90). LMP1 can be transmitted from lymphoid and epithelial cells in association with exosomes that also have immunosuppressive properties (111, 205).

In the lytic phase more than 60 proteins are produced with high copy numbers; which are highly immunogenic (see above). Consequently they require avoiding $CD8^+$ and $CD4^+$ T cells.

During millennia of co-evolution with its human host EBV has developed several ways to evade host immune responses in the lytic phase, similar as other herpsevisuses. BZLF1 expression is able to downregulate HLA class I 4-5 fold, and HLA class II 2-fold compared to EBV latent infection (203). In addition, the BZLF1 gene product can interfere with INF-γ-induced effects in the host cell (300). BCRF1 encoding vIL-10 is able to downregulate the expression of TAP-1 associated MHC class I antigen presentation and modulating T-cell responses away from cytotoxic activation, suppressing their ability to kill EBV-infected cells (263, 485). vIL-10 also prevents monocyte and macrophage to activate T cells (280, 359) as well as enhances survival of infected B cells by blocking IFN-γ response (420). Late phase gp42 binds to HLA class II peptide complexes creating new conformational at the surface of the molecule thus preventing $CD4^+$ T cell recognition (346, 347). Recently, BNLF2a protein showed to inhibit peptide transport function via binding to TAP and ATP binding functions, and reduce surface expression of HLA class I molecules. (159). BGLF5 (viral DNAse) was recently shown to induce shut-off of host protein synthesis, possibly by reduction of mRNA stability, thus down-regulating HLA class I surface molecule to present viral peptide to $CD8^+$ T lymphocytes (353, 493). BARF1 is naturally secreted from infected cells and may act as colony stimulating factor 1 (CSF-1) inhibit macrophage activation (411) and inhibit secretion of IFN-α in EBV infected B cells (65), whereas IFN-α plays role in early host response to viral infection. Thus, several EBV encoded functions contribute to immune evasion and aid to promote viral persistence.

5. Detection of EBV and its gene products

As EBV infects as many as 90% of total world population, it is important to discriminate healthy and disease state, and to differente different EBV-related diseases. Detection of EBV and its activity can be performed in several ways, as shown in figure 6.

Detection of DNA can be used to define the presence of EBV either in tissue, saliva or circulation. The earliest method to be developed was southern blot hybridization, currently being replaced by polymerase-chain reaction (PCR)-based EBV DNA amplification techniques. When compared to PCR, southern blot showed less sensitivity, is more complicated, requires more than 1% of target DNA to represent EBV (177). However, this method is still useful to detect EBV clonality in tumor DNA extracts (339). Real-time PCR which can quantify EBV copy number has replaced conventional PCR (402). Elevated number of EBV DNA may reflect the increase of EBV activity at present, either from body fluids, lymphocytes, or epithelial cells. Real time PCR is currently the preferred method for diagnosis, follow-up of therapy, and screening of high risk (403, 404). Rather than EBV-DNA load alone, the dynamic changes of load over time can be used as indicator for disease (406, 407).

EBV activity may also reflected by transcription of EBV DNA into RNA, which can be detected by reverse transcriptase PCR (RT-PCR) and NASBA (nucleic acid sequence based amplification). RT-PCR allows detection of small amount of RNA, by first transcript RNA to cDNA and amplifies by using conventional PCR. However parallel presence of DNA in sample gives complication and only spliced genes can be studied reliably (401). NASBA is an isothermal RNA amplification method to detect unspliced transcript even in the presence of background DNA. This method offers higher sensitivity and rapidity compared to RT-PCR (195, 204).

Morphological techniques may be applied to identify EBV-infected cell by the expression of EBV gene product. EBER-RISH (RNA in-situ hybridization) staining is the golden standard for EBV detection in tissue (8). This method is based on the abundant expression of EBER1,2 in most EBV positive infected cells. At present EBER-RISH is performed with non-radioactive labeling; still

this method is hampered by technical difficulties, and prone to mRNA degradation during sample preparation, thus may lead to false negative (294). On the other hand, immunohistochemistry employing monoclonal antibodies to EBV gene product ("markers") may replace EBER-RISH, for example EBNA1 (81), which presents in all tumor cells or LMP1 which is expressed in most latency-II tumors (193). Detection of latent (as well as lytic, when possible) genes product can help to determine latency type in single cell level (492). This method has offered options for detection of single or multiple EBV gene products, especially after antigen retrieval technique was established to unmask epitopes in paraffine sections masked by formaline fixation of tissue, providing more detailed information, and time efficiency (383).

Serology is the major method to determine status of EBV infection; by measuring antibody (ies) reactivity towards EBV antigen(s), distinct antibody pattern has been determined for individuals with primary infection, latent infected carriers and individuals at disease (152). Antibodies to individual EBV antigen can be measured by the immunofluorescense assay (IFA) on different cell substrates which is still the most widely used as golden standard for EBV serology (149). More recently enzyme-linked immunosorbent assay (ELISA), immunoblot detection, and

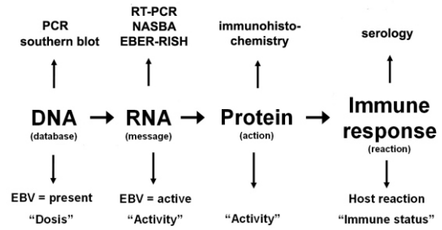

Fig.6. Detection of EBV in the DNA, RNA, and protein levels (with permission of JM Middeldorp)

neutralizing antibody methods have been used. Multiplex bead technique is a novel approach that allow simultaneous assesment of antibodies to multiple EBV antigens making EBV serology less labour intensive and providing standardization (6, 220). Molecular standardization of the EBV antigens used for serological testing is another issue to be achieved. Further details on EBV serology will be discussed in the experimental chapters of this thesis.

6. EBV-related diseases and malignancies

The association of EBV with benign and malignant diseases is unique among DNA viruses. Expression of different EBV latent genes suggest these diseases can be grouped into different latency types reflecting different pathogenic pathways (see table 1). The diseases can basically be divided into those occurring in immune suppressed individuals and those occurring in subjects without overt evidence of immune suppression.

6.1. Primary infection:

In developing countries, primary EBV infection mostly occurs asymptomatically during early life, leaving clinically asymptomatic individuals. However, delayed (adolescence) primary infection may lead to **infectious mononucleosis (IM),** a self-limiting lymphoproliferative disorder characterized by fever, sore throat, adenopathy and splenomegaly (229) with symptoms mainly caused by vigorous production of polyclonal CD8+ T cells. Heterophile antibodies (HA) consist of

IgM antibodies to sheep, bovine, and horse red blood cells and are produced by random EBV-transformed B-cells at early stage IM. This epiphenomenon was discovered in 1932 and still commonly used for diagnosis of IM, but hampered by low sensitivity and specificity as well as low level of antibody persisting for a year (417). For more accurate diagnosis, antibody measurement to specific EBV antigen should be used, demonstrating elevated IgM and IgG to EA and VCA (152). The anti-VCA IgG antibodies slowly rise during acute infection but persist for life. In contrast, the anti-VCA IgM and anti-EA(D) IgG antibodies rapidly decline as the patient recovers and are typically undetectable after 12 months (168, 321). IM usually resolves after 1-2 months, but may progress for more than 6 months to become chronic active infection ("chronic mononucleosis") with life-threatening complication over time. By immunoblot study, this disease showed high IgG response to EBV antigens, with reactivity towards IE, E and L antigens but not EBNA1 (96). This is considered as chronic disease, which may associate with development of B and T cell lymphoma (reviewed in (319).

EBV infection in immune suppressed subjects will lead to various diseases. The underlying cause ranges from inherited genetic defects on lymphocyte signaling (X-lymphoproliferative disorder (X-LPS) (373)], infection (oral hairly leukoplakia (OHL) and AIDS-related lymphoma (294)], and immune suppressive treatment (Post transplant lymphoproliferative disorder (PTLD) (165)).

X-lyphoproliferative disorder (X-LPS) is caused by defect in signaling lymphocyte activating molecule)-associated protein (SAP) (SLAM) (393). Primary EBV infection may lead to fulminant IM with virus-associated hemophagocytic syndrome (VAHS), due to defects in SAP molecules (229) .

6.2. Immunosuppression related disorders:

Oral hairy leukoplakia (OHL) is a benign disorders in immunosupressed subjects with acquired immunedeficiency syndrome (AIDS), characterized with white proliferative, hyperkeratotic, squamous mucosal epithelial cell lesion usually on the lateral side of the tongue. Abundant EBV particles are found in superficial epithelia (133), confirming ability for EBV to infect epithelial cell. In fact, OHL is a combination of latent and lytic infection where the latent protein (especially LMP1) provides growth and survival to the virus producing cell (458). Because normal EBV carriers only have sporadic EBV replication in the tongue (156), it is unclear how HIV-induced immunosuppression stimulates EBV lytic activation in the tongue Recent evidence points to a role of monocytes and DC in the pathogenesis of OHL (444). Acyclovir treatment may reduce the lesion, with possibility for OHL to regress into normal EBV persistence (451).

AIDS-related lymphoma (ARL) are heterologous B lymphocytic tumors derived from germinal centre or post-follicular B cells, and include CNS (central nervous system) lymphoma, diffuse large B cell lymphomas, Hodgkin's and non-Hodgkin's lymphoma (HL and NHL), BL-like lymphomas and primary effusion lymphomas, with variable frequency of EBV infection (229). CNS lymphoma showed presence of EBV in all cases, and most cases (> 60%) of diffuse large B cell-NHL and HL are EBV related, while BL and BL-like showed only 3-50% relation to EBV infection. It is considered that chronic antigenic stimulation in HIV-carriers may be at the basis of the increased frequency in lymphomagenesis. Except for OHL, most of the EBV related lymphoproliferative diseases under immune suppression will lead to lymphoma at later life if left unrecognized and untreated.

Post-transplant lymphoproliferative disease (PTLD) is an aggressive disease caused by proliferation of EBV positive B cells, facilitated by drug treatment for immunesuppresion. In solid

organ transplantation, EBV is often of donor origin, and the opposite is seen in bone marrow transplant recipient (422). Primary EBV infection causes most problems. Therefore it is important to determine EBV serostatus in donor and recipient, prior to transplantation. High incidence of PTLD was found when cyclosporin A (398) was first used as immunesuppressive drug, and diminished when drug dose was lowered (25). More recently EBV-DNA viral load measurement from whole blood fraction may be used to determine PTLD from acute cellular rejection showing similar symptoms, as well as for screening of PTLD (406). Regular screening for early stage PTLD may allow timely intervention and prevent fatal complications (293).

6.3. Malignancies in immunocompetent persons

EBV-associated cancers in immune competent subjects comprise Burkitt's lymphoma (BL), Hodgkin's Lymphoma (HL), several B-/T-/NK-cell non-Hodgkin's lymphomas (NHL), Gastric adenocarcinoma (GC) and nasopharyngeal carcinoma (NPC). In these malignancies, primary EBV infection occurs years before clinical onset; highlighting that other factors such as genetic, life style, inflammation and environment, may also play role in the carcinogenesis (294).

Burkitt's lymphoma (BL) is a pediatric tumor marked frequently with characteristic “starry sky macrophages” (23), occurs in malaria endemic area of equatorial Africa and Papua New Guinea, and shows 95% association with EBV infection (278), with increased IgG reactivity towards VCA prior to BL clinical development (78). EBV is present in a quiescent state, expressing non-coding EBERs and EBNA1 only. This malignancy characteristically shows chromosomal translocations of the *c-myc* oncogene on chromosome 8 and either immunoglobulin heavy chain locus on chromosom 14 (t8,14) or light chain loci on chromosome 2 and 22 (t8,2;t8,22), which alter its regulation and promoter arrangement (70). The chromosomal translocation may be triggered during gene rearrangement or class switching upon malaria-driven B-cell activation. CD10 and CD77, characteristic markers of BL, suggest BL as germinal centre B cell, which expanded during chronic malaria expansion (327). Interestingly, a surface protein on malaria parasites was recently shown to specifically activate B-cells, confirming a supportive role (58). BL in non endemic area (100 times less frequent) also showed translocation of *c-myc* and immunoglobulin, but at different breakpoints (348) with only 20-30% association with EBV as the infection occurs mostly at later age (278).

Hodgkin's lymphoma (HL) is characterized by presence of characteristic Hodgkin/ Reed-Sternberg (HRS) cells, comprising less than 1% of the tumor mass, and assumed to be derived from (post-)germinal centre B cells. When present, EBV is clonal and resides in HRS cells (229). Classical HL is divided into three subtypes: nodular sclerosis, mixed cellarity, and lymphocyte depleted, each showed association with EBV infection by 20, 70, and 100%, respectively. The higher EBV association coincides with poor prognosis of the disease (127). In less developed contries and in AIDS patients, almost 100% HD are related to EBV infection. Large epidemiological studies indicated that previous history of IM would increase the risk of HD (230). Aberrant EBV antibody responses preceed HD development, and have contributed to the understanding of the complex role of EBV in this disease (302).

Lymphoepthelioma-like carcinoma (LELC) sharing the same feature of abundant lymphoid infiltrates as undifferentiated nasopharyngeal carcinoma (WHO type III, see NPC discussion) were identified in various sites, including thymus, larynx, tonsil, salivary glands, lungs, skin, uterine, cervix, bladder and stomach. Tumors from the foregut-derived sites are endemic in certain population in the world (38, 173, 229, 235, 249, 464). A recent large sero-epidemiological study found that lymphomas lining the gastro-intestinal tract were more associated with abnormal EBV antibody responses than matched controls, although these tumors were largely EBV negative

(364).

About 10% of **Gastric carcinoma (GC)** worldwide is related to EBV irrespective of geographic origin (38, 384). GC is grouped into three different subsets, 10% of the gastric adenocarcinomas not otherwise specified (NOS) (424), >80% of the relatively rare lymphoepithelioma-like carcinomas (LELCs) of the stomach (38, 384), and 35% stump carcinomas (15, 471). EBV positive GC represents a distinct disease entity, differing from Helicobacter-associated GC, by localization, age, male predominance, immunological features and prognosis (19, 21). Gastric stump showed the highest association with EBV infection as many as 40% (471). An EBV causal role is yet to be defined, but EBV showed clonality in EBV-positive GC (178, 385), with as yet different latency pattern compared to other EBV-related malignancies (see table 1), showing the expression of BARF1 as putative alternative viral transforming factor (494). A high antibody titer to EBV was detected years prior to clinical diagnosis of EBV positive GC (120, 246, 387). EBV-positive GC has a better prognosis and shows a different location compared to EBV-negative GC, which is usually associated with Helicobacter pylory infection (20).

Extranodal NK/T-cell lymphoma (ENK/T) is an aggressive malignancy and frequently affects the nose and paranasal area (187, 323), with histological appearance of cucumber-like elongated nuclei. This tumor is most prevalent in SE-Asia and may relate to the higher frequency of chronic active EBV infection observed in this region. Pathologically this malignancy categorized into nasal type and aggressive lymphoma/ leukemia. (49). Diagnostical difficulty arises because paraffine-embedded tissue samples of NK and T lymphoma showed positive on CD3 staining, CD3 for T cell surface molecule, and CD3ε for cytoplasmic NK staining, resolved by staining on cryostat section (48). EBER-RISH staining found to be positive in the nasal type (60). EBV viral load is a useful tool to predict disease status (13), survival (170), but not as prognostic indicator (419). In SE-Asia chronic active EBV infection is more common and associates with hemophagocytic syndrome, frequently also involving T-/NK-cell lymphoma (180).

Other EBV-linked malignant diseases. The association between EBV and other malignant diseases deriving from cells other than B and mucosal epithelial cells, have been described based on PCR or staining of tumor cells. Leiomyoma and leiomyosarcoma of smooth muscle cell in children with HIV infection or (287) and transplant recipient (238) showed 90% positive in EBER staining and elevated number of EBV DNA copies in blood (189, 239). It was suspected that immunesuppression somehow enabled EBV to gain entry into into smooth muscle cells with still an unknown mechanism.

Controversial results were reported in EBV-related hepatocellular carcinomas (HCC) and breast adenocarcinomas. Examination of 35 HCC tissue samples of Japanese subjects showed 37% with positive EBV DNA by southern blot hybridization (414), but found to be EBER negative. However a study on a panel of hepatocellular carcinoma in European and North American patients could not demonstrate any evidence of EBV infections (197). Positive staining of “EBV-related” breast adenocarcinoma (31) was caused by cross reactivity between EBNA1 antibody (clone 2B4) with MAGE-4 cellular proteins, leading to false positive result (305). Nevertheless, small percentage of EBER-positive staining on breast adenocarcinoma was also reported (232, 285). Some of these findings may relate to common EBV infection in ductal epithelial cells in the breast (174).

EBV is also linked to initiation of autoimmune diseases, including Sjogren's Syndrome (SS), systemic-lupus erymathodus (SLE) and multiple sclerosis (MS), although the pathogenetic mechanisms remain speculative (139, 140).

7. Nasopharyngeal carcinoma (NPC)

NPC is a cancer with remarkable geographical and racial incidence, with annual incidence of less that 1/100,000 individual/year, with uneven distribution throughout the world. NPC represents only 0.25% of the cancers in Western populations including Hispanic, but its incidence is elevated in Eskimo population in Canada (281), Alaska (234), and Greenland (314), and in African and Arabs of the Mediterranean. It is one of the leading malignancy represents as many as 20% of the cases of cancer in southern China and Southeast Asia (339). The first report of NPC was found as early as 1901 (186), and a comprehensive study was reported with more patients in 1941 (86).

Cancer of nasopharynx is an epithelial cancer, frequently with abundant lymphocytic infiltrate, that occurs in the epithelial lining of the nasopharynx. Most EBV positive NPC arise from rossenmuller's, a fossa region rich in lymphoreticular tissue, and Eustachia cushion, or in the roof of naspoharynx but rare in the anterior and lateral walls (391).

Histopathologically, NPC is classified into three types based on degree of differentitation, i.e. keratinizing squamous cell carcinoma (WHO type I) highly differentiated and characterized by epithelial growth patterns and keratin fillaments, non-keratinizing squamous cell carcinoma (WHO type II) with retaining epithelial cell shape and growth pattern, and undifferentiated carcinoma (WHO type III) which does not produce keratin and lack of distinctive growth pattern (339). In endemic areas, undifferentiated type comprises the vast majority of NPC, while keratinized squamous cell carcinoma is less common (288). Worldwide, NPC WHO type II and III are associated with EBV infection in the tumor cells, whereas WHO I mostly is not.

Patients with NPC often presented with various types of symptoms, including one to four categories, (1) presence of tumor mass in the nasopharynx (epistaxis, nasal obstruction, and discharge); (2) dysfunction of Eustachian tube, associated with lateroposterior extension of the tumor mass to paranasopharyngeal space (tinnitus and deafness); (3) skull-base erosion and palsy of the fifth and sixth cranial nerves, associated with the superior extension of the tumor (headache, diplopia, facial pain, and numbness); (4) neck masses, usually appear first in the upper neck (460). Evaluation of 4768 NPC subjects by Lee et al. (1997) summarized the symptoms at presentation as neck mass (76%) which related to metastasis, nasal dysfunction (62%), headache (35%), diplopia (11%), facial numbness (8%), weight loss (7%)-- due to metastatic spread--, and trismus (3%). The physical signs present at diagnosis were neck node(s) (75%), and cranial nerve palsy (20%). Similar symptoms were found when comparing young and adult NPC subjects (241).

7.1. Staging, diagnosis and therapy

Staging of NPC is based on TNM classification, an anatomically based method, where T describes the primary tumor site, N regional lymph node involvement, and M describe distant metastatic spread (UICC site, 2007). There are 2 types of NPC classification of staging, Ho's and AJC/UICC (American Joints Committee/ (International Union Against Cancer) with 1997 revision. Ho's classification is mostly used in Asia including Hong Kong as the highest incidence region, while AJC and UICC are practiced more in USA and Europe (433).

Clinical examination including endoscopy provides information on mucosal involvement and tumor extension into nasal fossae and oropharynx, and CT scan helped to explore deeper to the cranial base. CT scan has identified paranasopharyngeal extension as one of the most common nodes of NPC extension (376). When compared to MRI, CT-scan is able to determine bone status, while MRI is more able to displaying superficial, soft tissue and marrow infiltration by tumors. Examination for distant metastasis should be done in high risk stage (N3) by including chest radiography, total bone scan, and liver ultrasonography (227).

NPC prognostic categories can be defined across several progression stages, i.e. stage

I: T1-2N0-1 (relatively good treatment outcome); stage II: T3-4 N0-1 (mainly local failure); stage III: T1-2 N2-3 (mainly regional and distant failure); and stage IV: T3-4 N2-3 (local, regional, and distant failure). The prognostic categories will help to determine appropriate treatment methods (63).

Radiotherapy is the standard initial treatment for NPC, with good success, and able to control Stage I-II with T3-4 up to 50-75% of cases (292), with interruption or prolonged treatment reducing the benefits of radiotherapy (231). To enhance the efficacy of treatment for NPC at later stage, radiotherapy is combined with chemotherapy, including neoadjuvant, concurrent, and adjuvant therapy with different results from each clinical trial. For treatment of local residual disease, brachytheraphy or phothodynamic therapy (PDT) may be used additionally (226, 468). *In vivo*, outgrowth of NPC tumor cells may be accompanied by acquisition of apoptosis resistance and

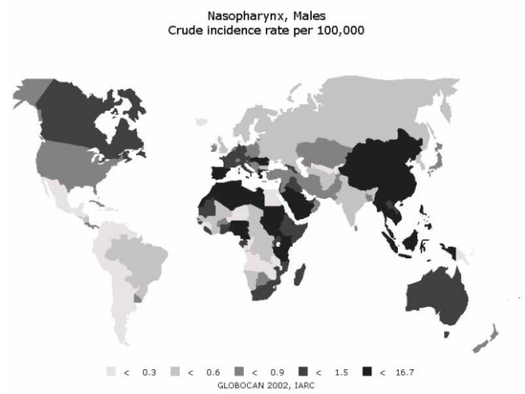

Fig.7. NPC incidence worldwide (male), showing unique distribution among different geographical area. Globally, NPC incidence in Indonesia is classified as intermediate incidence with less than 16.7 new cases/100.000/ year (CancerMondial; http://www.dep-iarc.fr/).

defective MHC-I expression, which allows tumor cells to escape cytotoxic T-cell responses (324). This phenotype is also under the influence of sensitivity to radio- and chemotherapy-mediated by apopotosis. EBV may be instrumental in this process, making cells to be apoptotic resistance (294).

7.2. Epidemiology

NPC is suspected to result from a combination of EBV infection, genetic susceptibility, (151), diet and environmental factors. Consumption of salted fish (11, 481), preserved food (483)-- with suspicious carcinogenic factor such as nitrosamine, formaline, butyric acid, and other co-factors, occupational exposure (480), and herbal drugs (488) are linked to NPC incidence. Host factors including association with defined HLA (Human Leucocyte Antigens) loci (52, 71, 130, 155), genetic and epigenetic abnormalities are also suspected to play a role (47, 104, 475).

NPC rates are higher in male than female with a ratio of 2-3: 1, with distinct age distribution across different populations (332, 482). In most populations, NPC is reported primary in adults (>30 years of age). For example, in Indonesia, especially in Yogyakarta province, analysis of 400 patients during 2001-2006 showed age distribution with median and mean of 46 and 45.5 years old, respectively (Fachiroh, unpubl. data). However, in Mediteranean populations a bimodal distribution was described (72, 207), with 20% peak incidence noted in juvenile (4-24 years old) and

a second peak in adults (40-60 years old).

Low socioeconomic status also plays a role in NPC incidence, as found in Southern China, indigenous people of South-East Asia, Arabs of North Africa and Inuits in polar region. Migration did not change incidence, as migrant keeps the traditional way of life. Therefore the incidence remains, with changes of incidence only seen after subsequent generations (484).

7.3. NPC and EBV

EBV is consistently detected in undifferentiated type of NPC (WHO type-III) from high and low risk incidence areas. NPC shows EBV latency type II, by having several EBV genes expressed in NPC biopsies, including EBERs, EBNA1, LMP1, LMP2A, and BARF1 (41, 80, 124, 161). Lytic phase antigen is not generally detected in tumor tissue, except low expression of BZLF1. This contrasts to high antibody responses in NPC which are directed to diverse early and late lytic phase antigens and also to EBNA1. These may reflect active viral replication in sub-epithelial sites, or as response to differentiating epithelial cell within the tumor, which then may switch on EBV lytic cycle (492).

Antibody profile to EBV antigens shows differences between juvenile and adult NPC. The antibody profile of juvenile (4-24 years old) NPC in Tunesia showed a more restricted pattern of IgG and IgA to EBV antigens compared to those of older age group (40-60 years old) (202).

Early seroepidemiology studies indicated that NPC as well as BL, showed elevated IgG antibody titers to EA and VCA antigens (153), and later studies defined especially IgA to both EBV antigens as a hallmark of NPC (147). Studies suggested that WHO type II-III NPC has 40-100% correlation with EBV infection, respectively, revealed by elevated titer of IgG ad IgA to viral EA and VCA antigens, while WHO type I shows normal IgG titer (224, 377). In addition EBV DNA was detected in poorly differentiated and undifferentiated NPC from high and low incidence areas (328), and not in squamous cell carcinomas representing type I. NPC WHO type I represents less than 1% of NPC cases in endemic area of high incidence, and less consistent findings of EBV DNA (339).

EBV-DNA in NPC biopsy material was first discovered by zur Hausen in 1970 (141), and can also be detected in cervical lymph node metastases (103). In support of relevance to EBV, premalignant lesions of the nasopharynx epithelium already harbor EBV, suggesting that the infection occurs at the early phase of carcinogenesis with virus-positive tumors containing genome that is typically “clonal” (136). Clonality is based on the fact that the EBV genome is flanked by variable numbers of terminal repeats (TR), with vary in number for each DNA molecule (see Fig.1.). Upon entry to the cell, a single viral DNA circularizes through the terminal repeat region (TR) to form a stable intracellular extrachromosomal episome. EBV DNA in individual tumor cells show identical TR length, indicating that NPC tumor derived from the same original infected cell (339). There is a complex link between EBV infection and NPC, essentially supported by observations that EBV infection is associated with faster growth, less differentiation, and more metastasis of NPC tumors, in comparison to non-infected cases (443). It is commonly recognized that EBV cannot infect untransformed squamous metaplastic epithelia (256, 257, 267). However, EBV infection is occasionally observed in NPC high-grade (transformed cells) but not in low-grade dysplastic epithelia, implying that some specific genetic changes are involved that precede EBV infection during NPC progression (267). The presence of EBV latent genes expression in normal tonsil epithelial explants was recently confirmed (175, 333). EBV may provide survival signals to chemically-damaged epithelial cells, thus driving cancer formation (294). Therefore it is now accepted that EBV infection is an early and necessary event in NPC pathogenesis.

7.4. EBV markers for NPC diagnosis

Current golden standard diagnostic procedure is the demonstration of EBER+ epithelial cells in an invasive biopsy taken from the nasopharyngeal space. IFA serology was developed early upon EBV discovery and used widely for detection of IgG/ IgA response to EA and VCA, and still is the gold standard for NPC serodiagnosis (245, 279). Application of both EBER-RISH and IFA methods for routine use is complicated by many difficulties (73, 95). Increases in circulating EBV-DNA levels, distinct features of EBV gene expression in the tumor cells in non-invasive brushings, and abnormal IgA antibodies to individual EBV proteins are features of NPC that may be exploited further (34, 50, 95, 146). It is suggested that some of these alternative techniques may replace EBER-RISH staining of the tumor cells, providing more easily accessible diagnostic testing in developing countries.

ELISA is an alternative method to replace IFA for measuring anti-EBV antibodies in serum/ plasma. This method provides a simple technique, may be applied for mass sampling and possible for automation. However, IFA detects antibody responses to intact EBV cells-containing a complex of EBV antigens smeared on a glass slide. Therefore, molecular identification of the right antigen is a challenge in order to develop a sensitive and specific ELISA system. Reported candidate antigens for ELISA include EBV cell extract (88, 89, 169), purified of native or recombinant protein (66, 73, 313, 389), and synthetic peptide (59, 74, 114, 202). Single and multiple EBV latent and lytic antigens were also reported to be immobilized in ELISA, i.e. EBNA1 (172, 202, 313), ZEBRA (73, 74, 389), ribonucleotide reductase (113), DNAse (408), EA-p138 (73), Major EA(D) (73, 202), gp350/220, VCA-p18 (172, 202, 313), were used in single or multiple ELISA assays aiming to improve overall sensitivity and specificity of NPC diagnosis (73, 202). So far, it is concluded that multiple antigen tests should be applied to achieve high sensitivity and specificity for NPC diagnosis. (topic of this thesis). The development of multiplex-multiparticle bead immunoassay may encompass the problem (220) by providing detection to multiple antigens in a single tube. Unfortunately, no data are available for NPC panel testing using this recent technology.

The presence of EBV DNA in tumor mass raises the possibility to detect DNA in circulation. Mutirangura et al. (308) showed the ability to detect EBV cell-free DNA from 75% of NPC panel tested. Lo et al. (270) developed real-time quantitative PCR assay based on *BamH1-W* region amplification for detection of EBV cell-free DNA in NPC patients. The method was able to detect EBV DNA in 97% of NPC and 7% of healthy individuals with much lower concentration compared to those of NPC subjects. Quantitative PCR by using other primer sets were also developed, e.g. pol-1 and LMP2 (237) showing similar result as *BamH1-W*-based amplification. EBV viral load from serum, plasma (46, 270, 307) or whole blood (403) are widely used for treatment monitoring (tumor burden) as well diagnosis confirmation accompanying serology (51, 100, 245, 268, 269, 447). Recent data showed that circulating EBV DNA is fragmented and represents material released from NPC cells via apoptosis and not relate to circulating tumor cells (403).

The NPC primary site is hidden, creating difficulty in biopsy and in-situ monitoring. Nasopharyngeal brushing (446) with nasoendoscopy guidance should offer a better (less invasive) sampling method. EBV DNA sampled from nasopharyngeal brush provides accurate information compared to cell-free circulating DNA (188, 310). Comparison of EBV viral load from cell-free and in situ samples method will be discussed further in this thesis.

Replacement of EBER-RISH by the development of immunohistochemistry staining based on EBV latent (and lytic) marker expressed in tissue has become common practice because of the availability of specific monoclonal antibodies to several EBV antigens. However lack of

standardization complicates consensus (37, 157, 206).

Among all latently type II genes expressed, only LMP1 and BARF1 considered as viral oncogene for their ability to induce malignant transformation ex-vivo (453, 459). BARF1 is exclusively expressed in EBV-positive carcinomas (NPC and gastric carcinoma) and absent from EBV-related lymphomas (33, 142). BARF1 protein is rapidly and efficiently secreted by epithelial cells (79, 360, 374), providing novel NPC diagnostic marker (169).

7.5. EBV-based assay for NPC screening

The five years survival steadily decreases with increasing NPC stage at presentation (394). At early stage (I-IIa), NPC is sensitive to radiotherapy showing 80-90% complete response and > 80% five years-survival which declines rapidly to 40% when treated at later stage (IIb-IV). Therefore detection in an early stage is important. This can be achieved by setting up a population-based screening in particular targeting a high risk population. High risk subjects include individuals with certain chronic head and neck symptoms (85, 191, 312, 394) and NPC familial history, especially in those in first degree (181, 271). Serology test is the most efficient and economical technique for epidemiology study, by the argument that elevated IgA response to EBV antigens may precede an early onset of NPC and serological tests are relatively cheap (191, 487).

Extensive NPC screening studies, involving 10.000-150.000 subjects, were done in high incidence areas, such China and Taiwan. Zeng et al. (487) reported a 4 years prospective study in city of Wuzhou, which included persons with high titer of IgA to VCA antigen at risk of having NPC. From 1136 individuals identified with high IgA titer, and 35 revealed NPC with 95% at stage I and II. The annual rate of this group was 31.7 higher compared to population as a whole. A 15 years prospective study on 40.000 individuals was done by Ji et al. (191) in China, with serial follow up including panels with low and high IgA titer to EBV antigens. Relative risk (RR) of low IgA/ EBV panel to develop NPC was 0.68, while those with high IgA/ EBV had RR of 5.81 (compared to population as a whole). This study was able to determine a "window period" of 16-41 months prior to clinical manifestation of NPC. Other seropidemiology study (61) presented similar result, with panel of high IgA/ EBV showed RR 22 times higher than the average population. Both studies (61, 191) were also identifying symptoms presented by those NPC subjects. Since most sero-epidemiological techniques were based on IFA method, attempt to develop a simpler serodiagnosis assay will be discussed in this thesis.

Application of EBV-DNA load from circulation (100, 380) or tumor cells (441, 446) was also proposed for NPC screening, because elevated EBV-DNA load in a normal healthy subject may suggests presence of early stage of NPC (380). Dynamic changes in EBV-DNA load may directly reflect disease progression; and was shown to offer higher sensitivity and specificity (100, 379) compared to IFA/ ELISA. However EBV-DNA load is still considered as expensive technology for developing countries. In addition a significant percentage of NPC patients may have low to undetectable EBV DNA in blood or plasma/serum (405).

7.6. NPC in Indonesia

In Indonesia, NPC is ranked as no.1. head and neck malignancy. Overall, NPC ranks no.4 in male and no.6 in females as most prevalent type of cancer. The available data mostly come from the Association of Indonesian Pathologist (hospital-based data). The data shows that in 2000 NPC incidence was considered to be intermediate (5.4/ 100.000 individual/ year), with regional hot-spots. Histopathologically about 70-80% of the cases were WHO type III. At diagnosis, most of patients presented with stage III-IV disease, which negatively affects cure rate and increases mortality rate. EBV-based NPC diagnosis is not used in daily practice, but mainly based on simple

HE staining of biopsy samples. Biological markers, DNA and/or antibody based diagnosis is not yet available due to high cost and lack of available laboratory space and trained personnel. Current commercial tests have disputable diagnostic value and are not optimized for the ethnical diverse target population in Indonesia. To down-grade NPC cases, a screening program needs to be prepared in order to find people with high risk and those with early stage of the disease, as well as to identify dietary and non-dietary habits related to NPC carcinogenesis. When related to other studies in China, Malaysia, and Singapore, salted fish, formaline preserved food, chemically dyed food, poor working/occupational environment, and smoking of local cigarettes (klembak-menyak, kretek) are suspected to linked with NPC (pers. comm. B. Hariwiyanto).

8. Aim of study

This thesis focusses on methods for NPC diagnosis and screening. We raised several questions:

Chapter 2: What is (are) predominant EBV marker protein(s) that might be useful for NPC serology?

Problem: EBV association with NPC is marked by diverse IgG and IgA responses to complex EA and VCA antigens.

EBV lytic antigens comprise of more than 80 proteins. Identification of immunodominant EBV marker(s) for NPC--discriminating NPC from healthy and non-NPC subjects--in Indonesia is needed. Here we determine which antigen(s) are useful candidates to formulate simpler yet sensitive and specific methods for NPC screening. By employing immunoblot-strips containing EBV- latent and lytic nuclear antigens separated by molecular weight we were able to identify IgG and IgA responses towards the individual EBV antigens in serum panels of Indonesian, Caucasian, and Chinese ethnical background. In the same time, we defined the diagnostic value of the system.

Chapter 3: Can we design a rapid and sensitive serological assay for NPC screening?

Problem: IFA is still a golden standard for NPC laboratory diagnosis.

The application of IFA is hampered by difficulties, and inconvenient for mass screening application. ELISA technique provides a promising alternative with potential for automation and easy handling of many samples at the same time. The key to develop a good ELISA system is the use of highly reactive EBV-specific antigen(s). Within this chapter we describe an ELISA system based on use of two chemically well-defined and cheap synthetic peptides representing immunodominant epitopes of EBNA1 and VCA-p18 antigens, and show its relevance for NPC screening. We propose to use this IgA/ [EBNA1+VCA-p18]-ELISA, for NPC screening in Indonesia.

Chapter 4: Can we improve EBV serology by an additional serological marker for NPC screening?

Problem: The peptide-based IgA/[EBNA1+VCA-p18]-ELISA misses about 10% of NPC population.

An additional marker(s) is required to achieve ~100% sensitivity. We have identified another potential marker to fill this gap, the p40 (BdRF1) antigen. This chapter shows the creation of a novel recombinant p40+p18 fusion protein that is used to develop an ELISA system. This new antigen is proposed to be added to the serological assay.

Chapter 5: Is there any simpler blood-sampling method for serological survey purposes?

Problem: The socio-economic and geographical situation in NPC high-risk populations with "distance" to referral hospital/lab require simple blood-sampling method to replace blood drawn from the arm.

Within this chapter we show the use of finger-prick dried-blood sampling and optimalization of antibody (IgA and IgG) eluted from dried blood on filter paper. This method (finger prick sampling

on filter paper) offers simpler application, easy transportation and handling, retains IgA antibody stability, efficient storage, and comparable IgA and IgG /[EBNA1+VCA-p18] reactivity compared to fresh plasma taken from the arm. Dried blood sampling is proposed to be used in combination with the IgA/ [EBNA1+VCA-p18]-ELISA in NPC field screening studies.

Chapter 6: Does EBV DNA from whole blood have diagnostic value for NPC screening?

Problem: an independent marker should be developed as confirmation for serology testing.

We have developed a real time PCR based on amplification of EBNA1 region from DNA extracted from whole blood. Result of the study disagree with other studies, and suggest that use of whole blood for EBV-DNA measurement is an inferior NPC diagnostic marker compared to serology. EBV DNA in circulation was derived from tumor cell apoptosis, and frequently negative in patients with viable (large) tumors. The use of circulating EBV DNA as NPC marker should be considered with care.

Chapter 7: Does EBV DNA load in-situ reflect biological activity of the tumor and does it provide non-invasive diagnostic value for NPC screening?

Problem: Biopsy is the golden standard for NPC diagnosis. This method requires an invasive biopsy which is difficult to perform and painful to the patient.

The use of nasopharynx brush offers a less invasive method for sampling in the area of the nasopharynx. From the brush, information can be obtained through different approaches, i.e. cytology staining and EBV DNA quantification and EBV RNA profiling. Our result show, better sensitivity and specificity compared to whole blood and may reflect direct biological activity of the tumor in situ. We propose to use this method as confirmation test to accompany serology in an NPC screening program.

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CHAPTER 2

Molecular Diversity of Epstein-Barr Virus IgG and IgA Antibody Responses in Nasopharyngeal Carcinoma: A Comparison of Indonesian, Chinese, and European Subjects

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ABSTRACT

Epstein-Barr virus (EBV) specific immunoblot analysis was used to reveal the molecular diversity of immunoglobulin (Ig) G and IgA antibody responses against Epstein-Barr nuclear antigen (EBNA), early antigen (EA), and viral capsid antigen (VCA) in serum samples from patients with nasopharyngeal carcinoma (NPC) and control subjects, by use of immunofluorescence assay (IFA). Control donors (n=150) showed IgG responses to few EBV proteins. VCA-p18, VCA-p40, EBNA1, and ZEBRA and sporadically weak IgA reactivity to EBNA1 and VCA-p18. Patients with NPC stage 1 (n=6) had similar response patterns. Patients with NPC stage 2-4 (n=132) showed significantly more diverse IgG and IgA responses to EA and VCA proteins -- VCA-p18/-p40--, EBNA1, Z-encoded broadly reactive activator, and EA(D)-p47/54, -DNAse,-thymidine kinase, and -p138. No correlation was found between IFA titers and the number of EBV proteins recognized by IgG or IgA. Our results reveal dissimilarity between EBV polypeptides recognized by IgG and IgA antibodies, which suggests independent B cell triggering events.

INTRODUCTION

Epstein-Barr virus (EBV), a γ -herpesvirus, is well established in the human population and is efficiently transmitted by mucosal secretions. EBV infection usually occurs silently early in life, but it may be symptomatic when infection is delayed until adolescence (1-3). EBV is also a human carcinogen that has been implicated in the development of malignancies of lymphoid and epithelial origin, including Burkitt lymphoma, Hodgkin disease (HD), immunodeficiency-related B cell lymphoma, extranodal T/NK cell lymphomas, gastric carcinoma, and nasopharyngeal carcinoma (NPC) (2, 4-6). Most EBV-associated malignancies show a distinct gene-expression pattern (7) and are accompanied by aberrant antibody responses to various EBV proteins and antigen complexes (8).

Undifferentiated NPC is 100% associated with EBV and forms an unusual tumor with intriguing epidemiological and biological characteristics (6-10). The highest incidence is found in persons of Chinese ethnicity living in southern China, Hong Kong, Taiwan, and Singapore. Intermediate incidence occurs in certain African and Mediterranean populations, in Inuits from Greenland and Alaska, and in Malays from Singapore and Malaysia. A low incidence is found in American and European whites, Hispanics, and Japanese. In Indonesia, NPC has an overall intermediate incidence (3.9 cases/100,000 population) similar to that in Malaysia. However, in the Yogyakarta area, NPC constitutes 21.8% of tumors in men and 7.9% of tumors in women, which ranks NPC as the most frequent tumor in men and the fourth most frequent tumor in women (11). The results of seroepidemiological studies have indicated a close relationship between EBV infection and NPC, as revealed by elevated IgG and, especially, IgA responses to EBV viral capsid antigen (VCA), early antigen (EA), and Epstein-Barr nuclear antigen (EBNA) complexes (8, 12). Elevated total and EBV-specific serum IgA levels are indicative of NPC stage (8, 13, 14) and can precede tumor development by 1-5 years, which suggests that a reactivation of EBV infection plays a role in tumor development (14, 15). In addition, a decline in anti-EBV antibody responses after radiotherapy may have prognostic value (16). At present, the indirect immunofluorescence assay (IFA) is still used as the reference standard for the serodiagnosis of EBV in NPC (2, 8, 12, 14). This method, however, is difficult to standardize and is not suitable for large-scale testing in developing countries; it is gradually being replaced by more-defined EIAs (17-20). Of importance, IFA does not provide insight into the molecular basis of anti-EBV responses, because EBV-infected cells each contain a multitude of different EBV proteins that can serve as the target or antibody interaction (21-23).

Recent molecular serological testing approaches in the diagnosis of NPC have focused on the use of defined recombinant EBV proteins. Tedeschi et al. (24) showed that antibodies against the Z-encoded broadly reactive activator (ZEBRA) protein are regularly found among patients with NPC. Others have proposed the anti-ZEBRA IgG antibody titer to be a prognostic marker for NPC (16). However, anti-ZEBRA IgG antibodies are detectable in 74% of healthy EBV carriers, according to the results of a sensitive immunoblot assay (22) -- this has been confirmed by exchanging blind serum samples (I. Joab and J.M.M., *unpublished data*). EBV DNAse neutralizing antibodies have been found in 83% - 94% of patients with NPC (25), and they appear to be a good marker for NPC screening and prognosis. However, there is no correlation between the level of anti-DNAse antibodies and antibody titer to VCA or EA(D), according to the results of IFA serological testing (26). Stolzenberg et al. (27) detected IgA antibody against recombinant DNAse in patients with NPC but rarely in patients with other EBV-related malignancies. Shimakage et al. (28) suggested the use of the EBNA1-IgA serum level as a prognostic marker for monitoring patients with NPC after radiation therapy, and Foong et al. (29) suggested the use of serum and salivary IgA levels against an EBNA1 Gly-Ala repeat peptide as a suitable NPC marker. Connolly et al. [20] suggested the use of thymidine kinase (TK) as the antigen in IgA-ELISA for the diagnosis of and screening for NPC. Dardari et al. (30) indicated that the combination of IgG-ZEBRA and IgA-EA [(p54)+(p138)] should be used. Most recently, Chan et al. (31) proposed the combined use of EBNA1-IgA and ZEBRA-IgG as the best predictor for NPC. It may be obvious from the above that there is no consensus on the use of defined EBV proteins in serological testing for the diagnosis and prognosis of NPC.

Still, very little detail is known about the overall molecular diversity (complexity) of antiEBV IgG and IgA antibody responses in patients with NPC (23). Moreover, a comparison of antibody profiles among patients with NPC who are of different genetic background has not been described. The study described here provides insight into the molecular basis of EBVspecific IgG and IgA antibody responses in patients with NPC of defined tumor stage from Javanese (Indonesia), Chinese (Hong Kong), and white (Europe) origin, compared with those in regional non-NPC control subjects and healthy EBV carriers. Our parallel analysis of IgG and IgA responses revealed differences in EBV antigen recognition profiles, which suggests independent B cell triggering.

SUBJECTS, MATERIAL, AND METHODS

Serum samples and antibodies. Serum samples from Indonesian Javanese (non-Chinese) subjects consisted of samples from a panel of 135 patients with histologically confirmed NPC, 5 patients with non-NPC head and neck cancer (all of which were collected at the Department of Ear, Nose, and Throat (ENT), Dr. Sardjito General Hospital, Yogyakarta), and 70 healthy donors obtained from the local Red Cross blood bank. The NPC serum samples were obtained on the first visit of patients to ENT during 2001 - 2003. From all patients with NPC, nasopharyngeal and/or lymph-node biopsy samples were obtained and confirmed histologically for the presence of undifferentiated carcinoma cells and the presence of EBV, by EBER1,2 *in situ* hybridization, by use of the Dako PNA-kit (Dako, Glostrup, Denmark) and by immunohistochemistry (Labvision) with EBNA1- and latent membrane protein (LMP)-1specific monoclonal antibodies OT1X (32) and OT21C (23, 33), respectively. NPC staging was done by ENT examination and computed tomography scan and was classified according to the 1997 Union International Cancer Control (UICC) classification.

Serum samples from persons of Chinese ethnicity living in Hong Kong were provided as a

blind panel and included samples from 40 healthy donors, 35 patients with head and neck-related non-NPC tumors, and 40 patients with histologically confirmed NPC (obtained by M.H.N.). The EBV serological profile of the Chinese panel was analyzed without knowledge of the clinical diagnosis. VCA and EA IgG and IgA antibody titers were determined by standard IFA techniques, according to the method of Henle and Henle (8), and this information was revealed only after breaking the code. Serum samples from 7 white patients with NPC were obtained from hospitals in Germany, the United Kingdom, and The Netherlands. One series (n=5) of follow-up samples from a white Dutch patient with NPC was obtained from the Vrije Universiteit medical center, Amsterdam, The Netherlands. All serum samples were stored at -20°C until use.

Monoclonal and polyclonal monospecific antiserum samples were produced by the immunization of animals with synthetic peptides or purified recombinant proteins, as described elsewhere. Antibodies to defined EBV proteins consisted of OT13B (anti-EA-p138; BALF2) (34), rabbit anti-EBNA1 (BKRF1) (35), rabbit anti-DNAse (BGLF5) (27), OT14E (anti-EA-p47;BMRF1) (36), BZ-1 (anti-ZEBRA; BZLF1) (37), OT41A (anti-VCA-p40; BdRF1) (38), and OT15E (anti-VCA-p18; BFRF3) (39).

Cell culture and antigen preparation. The superinducible P3HR1-derived cell line HH514.c16 was kindly provided by Dr. G. Miller (Yale University, New Haven, CT). Cells were cultured and induced for EBV lytic cycle antigen expression (EA only or EA plus VCA), and the nuclear fraction was prepared by hypotonic detergent treatment and Ficoll separation, exactly as described elsewhere (21-23). The EBV-negative cell line BJAB was used as a control.

SDS-PAGE and immunoblot analysis. The nuclear fractions were sonicated and boiled for 5 min in standard Laemmli lysis buffer and clarified by centrifugation at 14,000 g. Polypeptides were separated by SDS-PAGE in 10% acrylamide gels by use of the Bio Rad mini-gel system (Bio-Rad) and transferred onto 0.2-μm nitrocellulose membranes (Schleicher & Schuell) that were subsequently cut into 3-mm strips. Marker proteins (Bio-Rad Low MW Marker) were run on the side of the gel. Blot strips were immersed for 1 h in blocking buffer (5% [vol/vol] horse serum [Gibco BRL] and 5% [wt/vol] nonfat dry milk in PBS), to prevent nonspecific binding. For IgA detection, serum samples were treated with GullSorb (Meridian Diagnostic) to remove IgG antibodies, as described by the manufacturer. In all experiments, human serum samples were tested at 1:100 dilution in blocking buffer and incubated with the strips for 1 h at room temperature. Subsequently, the strips were washed 3 times with PBS that contained 0.05% Tween-20 (PBS-T), and horseradish peroxidase (HRP)-conjugated anti-IgG or anti-IgA antibody (DAKO) was added in appropriate dilutions and incubated for 1 h at room temperature. After washing 3 times with PBS-T and 2 times with PBS, bound HRP was visualized by 0.07% 4 chloro-1-naphtol and 0.01% (vol/vol) H_2O_2 in PBS. Stained strips were washed overnight with 10 ml of H_2O, dried, and stored in the dark until photography.

The position of characteristic EBV antigens was defined by monoclonal or polyclonal antibodies of known specificity (figure 1), which were detected with HRP-labeled anti-mouse or anti-rabbit antibodies (DAKO). In addition, from every batch of blot strips, 3 random strips were stained with reference human serum samples from a healthy seronegative and seropositive donor and serum from a patient with severe, chronic EBV infection (22, 23).

RESULTS

The P3HR1-derived HH514.c16 cell line can be induced to express high levels of lytic-phase EBV antigen (i.e., ZEBRA, EA, or VCA) on treatment with 12-O tetradecanoylphorbol 13- acetate and sodium butyrate. The use of phosphono acetic acid during induction effectively blocked the synthesis of EBV-DNA and the concomitant expression of late antigen (VCA), as revealed by the

absence of the VCA-p40 (BdRF1) and VCA-p18 (BFRF3) marker proteins (figure 1A). The results of previous cell fractionation studies have shown that diagnostically relevant antigens mainly reside in the nuclear fraction of both EA and EA-plus-VCAinduced cells (21, 22).

Reference antibody staining. The position of EBV marker proteins on the blot strips was defined by use of a panel of antibodies of defined specificity. Figure 1A shows the position of EA(d)-p138 (BALF2; 138 kD), EBNA1 (BKRF1; 72 kD), DNAse (BGLF5; 55+57-kD doublet), major EA(D) (BMRF1 4754-kD diffuse smear), VCA-p40 (BVRF2+BdRF1; 50+40-kD sharp bands), ZEBRA (BZLF1; 36+38-kD fine doublet), and VCA-p18 (BFRF1; 20 kD) on strips that contained EA and VCA. The position of EA-TK just below EBNA1 was revealed in a previous study (22). On strips that contained EA only, the VCA-p18 and VCA-p40 bands were absent, but EA and EBNA1 bands were detectable. EBV marker proteins were defined on the basis of size, staining pattern (doublet,

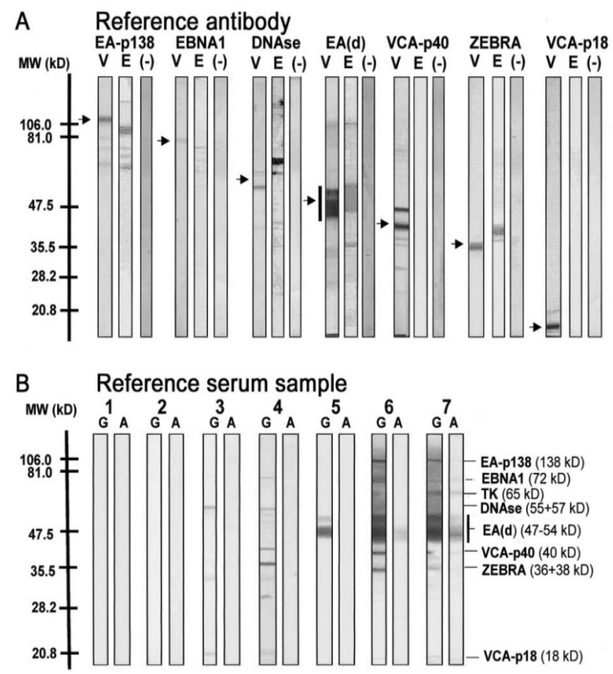

Fig. 1. Immunoblot reactivity patterns with reference antibodies. (A), Antigen applied to the blot: polypeptides from the nuclear fraction of HH514.c16 cells induced to express early antigen (EA) and Epstein-Barr virus (EBV) viral capsid antigen (VCA) (V, VCA induced), EA only (E, EA induced), and an EBV-negative cell line (_, BJAB). The monoclonal antibodies used in panels AC are specific for EA-p138, Epstein-Barr nuclear antigen (EBNA1), DNAse, major EA-D p47/54, VCA-p40, Z-encoded broadly reactive activator (ZEBRA), and VCA-p18, as detailed in Subjects, Materials, and Methods.
(B) Characteristic IgG (G) and IgA (A) reactivities for a set of human reference serum samples from EBV-negative donors (1 and 2), healthy EBV carriers (3 and 4), a patient with mononucleosis (5), a patient with severe chronic EBV infection (6), and a patient with stage 4 nasopharyngeal carcinoma (7). MW, molecular weight.

diffuse, or sharp band), and EBNA, EA, and VCA characteristics. Strips that contained BJAB nuclear extract did not show any bands. In all experiments, human reference serum samples were used as controls (figure 1B). These included 2 EBV-negative serum samples as specificity controls (samples 1 and 2); 2 serum samples from healthy seropositive donors (samples 3 and 4), to represent "normal" staining patterns observed in most healthy carriers worldwide (22, 23); an infectious mononucleosis serum sample the characteristic dominant EAd-p47/54 band (21) (sample 5); a serum sample from a patient with chronic, severe EBV infection (sample 6); and a sample from a patient with NPC (sample 7), to represent "strong" positive staining control. These serum samples reproducibly gave identical banding patterns and staining intensities on different batches of EA and EA-plus-VCA blot strips.

EBV-antigen recognition pattern in healthy EBV carriers. Healthy blood donors from Indonesia (n=70) and control subjects (n=5) showed a highly restricted IgG reactivity pattern, as was previously found in white persons from Europe and the United States (21, 22) (figure 1B, lanes 3 and 4), that were characterized by dominant responses to EBNA1 (BKRF1; 72 kD) and VCA-p18 (BFRF3; 18 kD), with occasional weaker responses to VCA-p40 (BdRF1, 40+45 kD) and ZEBRA (BZLF1; 3638 kD). IgA-EBV reactivity was absent in all samples except in 1 Indonesian control subject with non-NPC cancer, who showed weak IgG and IgA recognition of the EA-p47/54 (BMRF1) and VCA-p40 (BdRF1) proteins (data not shown). This individual had World Health Organization class 1 squamous carcinoma, and the tumor tested negative for all EBV samples at NPC stages 2, the most distinctive IgG reaction, compared with control samples, was directed against the early antigens EA(d)-p47/54 (BMRF1), DNAse (BGLF5), and TK (BXLF1) protein (figures 2 and 3). The increasing diversity of antibody responses against EBV proteins at higher stages of malignancy reflects viral replication that is associated with NPC tumor growth.

Comparison of IgG and IgA reactivity patterns in patients with NPC. Parallel analysis of antigen-recognition patterns for IgG and IgA antibodies in serum samples from the Hong Kong and Indonesian patients with NPC were done. Direct comparison of the individual NPC serum samples revealed a clear overall dissimilarity of EBV antigens recognized between IgG and IgA antibodies, as shown in figure 2. IgA reactivity does not seem to increase significantly with NPC stage, as was observed for IgG, but is, rather, more variable among individuals. Although IgA reactivity to EBNA1 and VCA-p18 was most frequently observed, the distribution and intensity of additional IgA reactive bands varied considerably among patients. For instance, some serum samples with strong IgG responses to multiple EBV proteins showed hardly any IgA response, as revealed by NPC 27 and 31 from the Indonesian panel (figure 2). The reverse situation, with dominant IgA reactivity, was found in patients 3 and 8 with NPC in the Indonesian panel (figure 2). Although IgG against ZEBRA was frequently detectable, IgA reactivity to ZEBRA was not predominant in the Hong Kong and Indonesian NPC groups. Overall, IgA responses frequently displayed a different pattern than IgG in the same sample. This implies that IgG- and IgA-producing B cells are triggered by different antigens or antigen fragments (epitopes), possibly at different locations in the body.

Comparison of IFA antibody titer and immunoblot detection. Table 1 shows an overview for the serum samples from patients with NPC from Hong Kong of IgG and IgA antibody titers to EA and VCA, as determined by routine IFA testing, in combination with a listing of the major EBV-specific antigen bands for each serum, defined by IgG and IgA immunoblot. The overall data showed a lack of correlation between IFA titer and immunoblot reactivity pattern to individual EBV proteins for either an IgG or IgA response. In some serum samples, high titers in IFA (e.g., NPC 25) were related to antibody recognition of only a limited number of EBV proteins, whereas, in other

serum samples with similar titers, the results of immunoblot revealed the recognition of multiple EBV polypeptides (e.g., NPC 9). In reverse, some serum samples with low IFA titers (e.g., NPC 31) bound to multiple EBV proteins, as revealed by immunoblot analysis. This result is in agreement with those of recent studies that compared EBV recombinant line-blot and IFA results (40) and

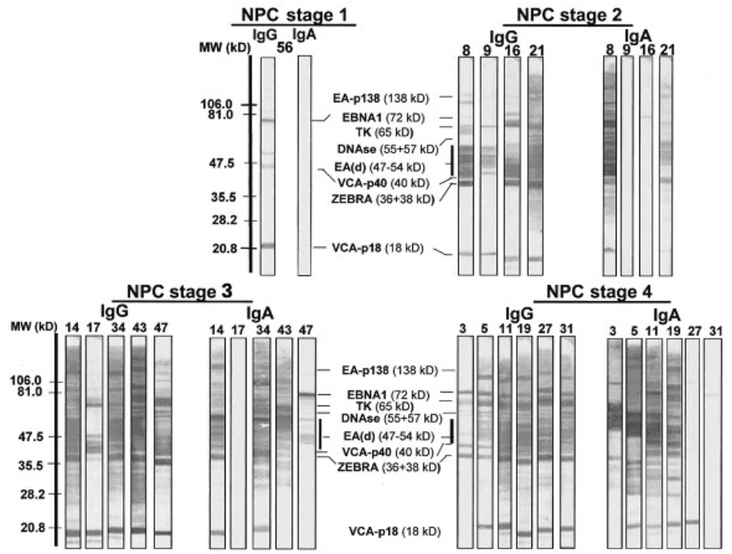

Fig 2. Immunoblot analysis of IgG and IgA responses in Indonesian patients with nasopharyngeal carcinoma (NPC). A characteristic set of IgG and IgA immunoblot profiles is shown for samples from the Indonesian NPC panel, classified according to tumor stage. Epstein-Barr virus (EBV) antigens applied to the blot consisted of nuclear fraction of HH514.c16 induced to express EBV early antigen (EA) and viral capsid antigen (VCA) proteins. All serum samples were tested at 1:100 dilution. EBNA, EBV nuclear antigen; MW, molecular weight; TK, thymidine kinase.

clearly reflects that IFA titers provide only limited information about the true diversity of anti-EBV responses in patients with NPC.

Follow-up case. In figure 4B, the follow-up analysis of a white patient with stage 4 NPC is presented. Sampling started at the end of combined chemoradiation therapy and continued at 3-month intervals for 15 months. In this patient, dominant IgG responses were directed against the TK and ZEBRA proteins and relatively minor, but diagnostically significant, responses to EA-p138, DNAse, and EAd-p47/54 proteins. The overall IgG diversity pattern in this patient remained stable over time but showed a gradual reduction in staining intensity, which reflects a waning antibody response to EBV lytic proteins (VCA and EA). This was paralleled clinically by complete clinical remission after 15 months of follow-up.

DISCUSSION

Table 1. Comparison of Epstein-Barr virus (EBV) early antigen (EA) and viral capsid antigen (VCA) IgG and IgA antibody titers, measured by immunofluorescence assay (IFA), and dominant IgG and IgA reactive EBV EA and VCA polypeptides defined by immunoblot, by use of samples from Chinese patients with nasopharyngeal carcinoma (NPC) and control subjects from Hong Kong.

Patient no., Stage	IF-IgG, EA	EA immunoblot					IFA-IgA, VCA	VCA immunoblot			
		Zebra	p47/54	DNAse	TK	p138		p18	p40	p160	
NPC 1											
10	40	+	+	+	+	+	160	+	+	+	-
15	40	+	+		-	+	640	+	+	+	+
25	10	+	+		+	+	640	+	+	+	
33	40	+	+	+	+		2560	+	+	+	+
39	10	+	+	+	+	+	640	+	+		
NPC 2											
7	40	+	+	+	+	+	160	+	+	+	
8	10	+	+	+	+	+	640	+	+		
18	< 5	+	+	+	+		160	+	+	-	
19	40	+	+	+	+	+	640	+	+	+	+
20	640	+	+	+	+	+	2560	+	+	+	+
22	160	+	+	+	+	+	2560	+	+	+	+
23	40	+	+	+	+	+	2650	+	+	+	+
31	40	+	+	+	+	+	640	+	-	+	+
37	40	+	+	+	+	+	640	-	+	+	+
NPC 3											
1	40	+	+	+	+	+	640	+	+	-	
4	40	+	+	+	+	+	640	+	+	+	
6	640	+	+	+	+	+	2560]	-	-		
9	160	+	+	+	+	+	2560	+	+	+	
12	10	+	+	+	+	+	640	+	+	+	+
13	40	+	+	+	+	+	640	+	+	+	+
14	160	+	+	+	+	+	2560	+	+	+	+
32	10	+	-	+	+	+	640	+	+	+	
34	640	+	-	-	+	+	640	+	+	-	
NPC 4											
11	640	+	+	+	+	+	640	+	+	+	+
21	40	+	+	+	+	+	2560	+	+	+	+
24	10	+	+		+	+	640	+	+	+	+
26	160	-	+	+	+		2560	+	+	+	+
29	40	+	+		+	+	640	+	+	+	
35	40	+	+	+	+	+	2560	+	+	+	+
Control subjects											
2	< 5	+	+	+	+		640	+	+	+	
5	5	+	-				160	+	+	+	
17	40	+			+		640	+	+	+	
38	< 5	-					160	+	+	+	

NOTE IFA titers are expressed as reciprocal serum dilutions. Immunoblot reactivities are scored (+ or -) for reactivity with the polypeptide identified by name (for EA, Zebra, p47/54, DNAse, Thymidine kinase [TK], and p138; and for VCA: p18, p40, and p160)

EBV-associated diseases are characterized by distinct antibody patterns to various EBV-determined antigen specificities, as defined by IFA serological testing [8, 41]. Aberrant levels of EA and VCA-reactive IgG and IgA antibodies can be detected in the serum and saliva of patients with NPC at early stages of the disease (8, 12, 19, 29, 42). The precise location of the NPC related EBV lytic gene expression that triggers antibody responses remains undefined. Zhang et al. [36] showed that EA and VCA expression associates with sporadic epithelial cell differentiation within the NPC tumor, which might trigger characteristic IgA antibody responses, but additional sites of lytic EBV replication may exist. Murphy (43) proposed that serum IgA results from spillover at mucosal sites, but IgA-memory B cells of mucosal origin might spread systemically, and their stimulation may yield systemic IgA.

At present, the overall molecular diversity of systemic EBV specific IgA responses is rather unexplored, and a direct comparison of the molecular fine specificity of NPC-related IgG and IgA responses in patients of different geographical and ethnical origin is lacking. Previous studies have addressed molecular aspects of EBV serology for the diagnosis of NPC, by use of single purified proteins or related peptides, such as DNAse (25-27), EA-D-p138 (44), EA-(D)-p47/54 (17, 30), TK (20), ZEBRA (16, 30, 31, 45), EBNA1 (17, 19, 28, 31), and VCAp18 (19, 29). In the present study, we used the immunoblot technique (21, 22), which allows side-by-side analysis of IgG and IgA reactivity against nearly the full spectrum of EBV proteins. Our results in Southeast Asian blood donors extend previous findings in whites from Europe and the United States, which have shown that healthy EBV carriers and patients without EBV-linked diseases have a highly restricted IgG antibody diversity, regardless of their geographic origin (21-23, 3)]. EBV reactive IgA was not detected in most EBV healthy carriers, except for an occasional response to either VCA-p18 or EBNA1. This uniform response to a limited set of EBV proteins reflects the well-balanced virus-host relationship (22). Compared with those in healthy carriers, significantly different diversity patterns are found in patients with acute and chronic EBV syndromes, including infectious mononucleosis (2123, 37), HD (46), and NPC (present study). The antibody-recognition pattern in patients with NPC differs from that in patients with other EBV syndromes and reflects the distinct underlying viral activity in NPC. Of importance, similar IFA antibody titers in different EBV-associated diseases may represent different antibody diversity patterns, the latter of which more directly reflect different EBV involvement. Thus, the immunoblot system provides a more detailed insight into virus-host interaction in different disease syndromes. The overall EBV-specific IgG antibody reactivity tends to increase with NPC stage, in line with the results of previous serological studies that have used IFA testing (6,), but the IFA titer does not reflect the underlying antibody diversity. Patients with stage 1 NPC showed restricted responses, largely without IgA, that were comparable to those of healthy EBV carriers and patients with non EBV-related malignancies. Although EBV is involved in the early and premalignant stages of NPC (6), this may proceed without keratinization and lytic gene expression and, thus, not trigger IgA EA and VCA antibody responses (36). The lack of reactivity to EA-R-p17 (a BHRF1-encoded bcl-2 homologue) is in agreement with the results of previous IFA studies [8] and with recent data on the expression of BHRF1 in NPC (4). Patients with NPC have rather limited antibody responses to the tumor associated latent membrane proteins LMP1 and LMP2 (23, 46, 48), as has also been found in HD (48). However, EBNA1 protein released from lysed NPC tumor cells induces strong anti-EBNA1 responses. The biological and immunological basis underlying different immune responses to individual EBV proteins remains a subject for further study.

It is generally assumed that IgG and IgA responses to EBV proteins have similar antigen reactivity. However, our results indicate clearly that IgG and IgA responses are triggered differently. Multiple examples in figures 2 and 3 reveal that IgA and IgG in the same serum bind to different sets

of EBV proteins. In addition, no correlation was found between IFA titer and the number of EBV protein bands identified by immunoblot (table 1), which indicates that both techniques detect different sets of antibodies binding to different EBV-encoded proteins or epitopes. Immunoblot reactivity more directly reflects the biological activity of EBV in patients with EBV-linked diseases. As is shown in figure 4, a white patient with a complete clinical response after combined chemoradiation therapy showed decreasing antibody reactivity over period of 15 months. Thus, antibody profiling by immunoblot may be used as a prognostic marker.

In conclusion, our results show that the molecular complexity underlying anti-EBV antibody responses in patients with NPC differs significantly from that of healthy EBV carriers and patients with non-NPC cancer. The antigen-recognition patterns of both IgG and IgA increases with NPC stage, most significantly stage 2. The EBV antigen diversity of IgG and IgA varies considerably between individual patients with NPC and seems to be driven by different antigen-triggering events. The EBV immunoblot diversity pattern has significant value for discriminating between NPC and non-NPC tumors and provides valuable information for the development of molecularly defined

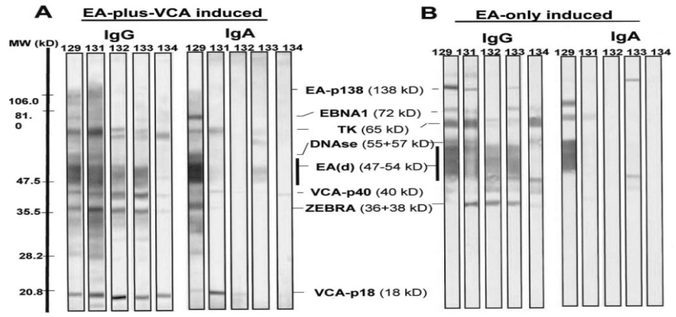

Fig. 3. Comparison of immunoblot reactivities of IgG and IgA responses to HH514.c16 induced to express Epstein-Barr virus (EBV) viral capsid antigen (VCA) plus early antigen (EA; A) and EA-only (B) polypeptides. Serum samples applied to the blot are consecutive samples from the Indonesia panel. All serum samples were tested at 1:100 dilution. EBNA, EBV nuclear antigen; MW, molecular weight; TK, thymidine kinase

EBV serology.

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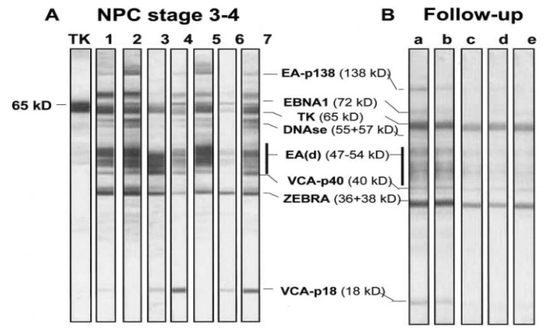

Fig. 4. Immunoblot analysis of IgG responses in the white nasopharyngeal carcinoma (NPC) panel. A, IgG response to viral capsid antigen (VCA; plus early antigen [EA]) proteins in patients with stage 34 NPC (17). Rabbit anti thymidine kinase (TK) was used as a marker. B, Longitudinal IgG responses of a white patient with NPC who showed good clinical response to treatment, sampled at 3-month intervals after treatment, for 15 consecutive months. Epstein-Barr virus (EBV) antigens applied to the blot consisted of nuclear fraction of HH514.c16 induced to express EBV EA and VCA proteins. All serum samples were tested at 1:100 dilution. EBNA, EBV nuclear antigen

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CHAPTER 3

Single-Assay Combination of Epstein-Barr Virus (EBV) EBNA1- and Viral Capsid Antigen-p18-Derived Synthetic Peptides for Measuring Anti-EBV Immunoglobulin G (IgG) and IgA Antibody Levels in Sera from Nasopharyngeal Carcinoma Patients: Options for Field Screening

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ABSTRACT

Assessment of immunoglobulin A (IgA) antibody responses to various Epstein-Barr virus (EBV) antigencomplexes, usually involving multiple serological assays, is important for the early diagnosis of nasopharyngeal carcinoma (NPC). Through combination of two synthetic peptides representing immunodominant epitopes of EBNA1 and viral capsid antigen (VCA)-p18 we developed a one-step sandwich enzyme-linked immunosorbent assay (ELISA) for the specific detection of EBV reactive IgG and IgA antibodies in NPC patients (EBV IgG/IgA ELISA). Sera were obtained from healthy donors (n=367), non-NPC head and neck cancer patients (n =43), and biopsy-proven NPC patients (n = 296) of Indonesian and Chinese origin. Higher values of optical density at 450 nm for EBV IgG were observed in NPC patients compared to the healthy EBV carriers, but the large overlap limits its use for NPC diagnosis. Using either EBNA1 or VCA-p18 peptides alone IgA ELISA correctly identified 88.5% and 79.8% of Indonesian NPC patients, with specificities of 80.1% and 70.9%, whereas combined single-well coating with both peptides yielded sensitivity and specificity values of 90.1 and 85.4%, respectively. The positive and negative predictive values (PPV and NPV, respectively) for the combined EBNA1 plus VCA EBV IgA ELISA were 78.7% and 93.9%, respectively. In the Indonesia panel, the level of EBV IgA reactivity was not associated with NPC tumor size, lymph node involvement, and metastasis stage, sex, and age group. In the China panel the sensitivity/specificity values were 86.2/92.0% (EBNA1 IgA) and 84.1/90.3% (VCA-p18 IgA) for single-peptide assays and 95.1/90.6% for the combined VCA plus EBNA1 IgA ELISA, with a PPV and an NPV for the combined EBV IgA ELISA of 95.6 and 89.3%, respectively. Virtually all NPC patients had abnormal anti-EBV IgG diversity patterns as determined by immunoblot analysis. On the other hand, healthy EBV carriers with positive EBV IgA ELISA result showed normal IgG diversity patterns. By using EBV IgG immunoblot diversity as confirmation assay for EBV IgA ELISA-positive samples, the sensitivity and specificity for NPC diagnosis increased to 98% and 99.2%, respectively, in the Indonesian NPC samples. The use of these combined methods for seroepidemiological screening studies is proposed.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a highly prevalent malignancy in southern China, most of Southeast Asia, and north Africa (36, 48). In Indonesia, especially in central Java, undifferentiated NPC (WHO type III) also ranks among the most common cancers. For instance NPC is ranked 1 in males and 3 in females in the Yogyakarta province (38), with regional villages representing hot spots of NPC incidence.

The consistent expression of Epstein-Barr virus (EBV) gene products in NPC tumor cells (33, 36, 47, 48) and the distinct serological responses to defined EBV antigens in NPC patients illustrate the close association between EBV and this disease (18, 19, 37, 49). The mucosal origin of NPC is reflected by characteristic immunoglobulin A (IgA) responses in NPC patients (13). Of the latency-associated EBV gene products expressed in NPC tumor cells only EBNA1 induces strong IgG and IgA antibody responses; LMP1, LMP2, and BARF1 do not (28, 42). On the other hand NPC patients have strong and characteristic antibody responses against EBV lytic-cycle proteins, including early (EA) and viral capsid (VCA) antigen complexes. Lytic-cycle products are rarely expressed in the NPC tumor cells but may originate from viral replication in differentiating NPC cells (51). The assessment of anti-EBNA1, anti-EA, and anti-VCA antibodies requires separate assays, each contributing to NPC diagnosis (23). Individual NPC patients may respond quite differently to EBV lytic-cycle proteins, but the small capsid protein VCA-p18, encoded by the BFRF3 reading frame, is a highly prevalent target for antibody responses, including IgG and IgA (3, 9, 13, 23, 35,

44). However, high titers of IgG against EA/VCA are not specific for NPC and can be observed in other EBV-linked diseases as well (18). Elevated IgA antibodies against EA/VCA and nuclear antigens, especially to EBNA1 (collectively referred to as EBV IgA), present an outstanding feature of NPC patients (10, 16). The molecular diversity of EBV antigens recognized by IgG and IgA antibodies differs between individuals and increases with tumor stage. Moreover the diversity of antigen recognition between IgG and IgA responses within an individual NPC patient may also differ significantly, suggesting independent triggering of IgG- and IgA producing B cells (13). Importantly, the presence of EBV IgA antibodies is associated with increased NPC risk in the general population (6, 10, 50). Therefore it was suggested that field screening for EBV IgA might be useful to identify patients with early-stage NPC (50). In addition, in established NPC patients, longitudinal monitoring of EBV IgA reactivity levels may be used for prognosis, because declining reactivity is associated with remission and stable or increasing responses are associated with persistent or recurrent disease and development of metastasis (34).

Currently indirect immunofluorescence assay (IFA) methods are still widely used as the “gold standard” for EBV serodiagnosis in NPC (24, 36). IFA, however, is time-consuming, not well standardized, and ill suited for large-scale testing or automated handling. Enzyme-linked immunosorbent assay (ELISA) techniques provide a promising alternative with potential for automation and mass screening However, ELISA methods described to date have used a variety of EBV antigens, and standardization has not been achieved yet (17). Standardized EBV IgA ELISAs suited for field screening require the availability of high-quality, reproducible, and preferably cheap EBV antigens comprising the immunodominant markers for EBV IgA antibodies. Previously reported EBV antigens for ELISA include EBV cell extracts (11, 12, 46), purified native or recombinant EBV proteins (3, 7, 9), and synthetic peptides (16, 23, 44). Among the defined EBV antigens proposed as markers in ELISA are thymidine kinase (7, 25), DNase (41), ribonucleotide reductase (15), ZEBRA (9, 22), VCA-p18 (35, 44), EBNA1 (16, 21), and EA(D)-p47/p138 (14). However, single markers may not be sufficient to identify all individual NPC patients in view of the observed diversity of antibody reactivity among individual NPC patients. Combined testing for ZEBRA and VCA-p18 IgA or ZEBRA plus EBNA1 IgA reactivity was recently proposed for more-sensitive NPC diagnosis (1, 2, 9). In all cases two-marker testing required testing by two separate assays.

A single assay combination for simultaneous analysis of IgA/ IgG antibody responses to multiple EBV proteins is provided by immunoblot testing using either extracts from EBV producer cells (13) or combinations of recombinant proteins as used in commercial line blot assay (17). More recently a combination of EBV IgA serology and EBV DNA quantification was proposed (2, 24, 26). However, these assays are laborious and expensive and therefore not well suited for mass screening.

In this study we describe the development of an IgA ELISA for the primary diagnosis of NPC by combining EBNA1- and VCA-p18-derived multiepitope synthetic peptides in a single well format. Synthetic peptides have the advantage of being chemically defined, allowing reproducible large-scale production at low costs and improved assay standardization (45). The combination of two immunodominant antigens for EBV IgA detection holds promise for development of a simple NPC screening and monitoring assay. EBV immunoblot IgG diversity analysis, recently shown by us to provide detailed molecular and diagnostically relevant information for NPC serology, was used as a confirmatory assay (13).

MATERIALS AND METHODS

Sera. The Indonesia serum panel consisted of sera from 147 histologically confirmed

NPC patients, 43 non-NPC patient controls (22 with other head and neck cancer, 21 with breast cancer), all collected at Sardjito General Hospital, and from 254 healthy donors, obtained from the local Red Cross blood bank (n=180) and hospital/university staffs (n=74) at Gadjah Mada University, Yogyakarta, Indonesia. The NPC sera were taken on the first visit of patients to the ear, nose, and throat department (ENT) during the years 2001 to 2003. NPC staging was done by ENT examination and computed tomography scan and classified according to the 1996 Union International Cancer Control classification. From all the NPC patients that enrolled at ENT, postnasal and/or lymph node biopsy samples were obtained and confirmed for the presence of undifferentiated carcinoma cells and the presence of EBV by EBV-encoded small RNA in situ hybridization using a peptide nucleic acid probe (Dakopatts, Glostrup, Denmark) or by immunohistochemistry (Labvision Corp., Fremont, CA) using EBNA1- and LMP1-specific monoclonal antibodies OT1X (5) and OT21C (28, 29) or both tests.

The China serum panel consisted of sera from 183 confirmed NPC patients and 113 regional healthy controls from south China (10). A panel of sera from infectious mononucleosis (IM; n=39) and chronic EBV (C-EBV) patients (n=10) and healthy donors (n=6) from The Netherlands were used as controls from a nonendemic area. These reference sera were obtained from the archives of the VU University Medical Center in Amsterdam, The Netherlands (28). All sera were stored at -20^{0}C until use.

EBV synthetic peptides. Immunodominant epitopes on VCA-p18 and EBNA1 were defined as described before (31, 32, 44). Briefly, from the predicted amino acid sequence encoded by the BFRF3 (VCA-p18) and BKRF1 (EBNA1) open reading frames on the EBV genome, all possible 12-mer peptides with an overlap of 11 were synthesized on polypropylene pins and tested by PEPSCAN analysis as described by Middeldorp and Meloen (31). Immunodominant epitopes for VCA-p18 (BFRF3) were located at amino acids 119 to 148 and 153 to 176 (44), and for EBNA1 (BKRF1) such epitopes were located at 382 to 410 and 413 to 452 (32). Individual 30-mer polyepitope peptides spanning the respective sequence were synthesized by 9-fluorenylmethoxy carbonyl chemistry, purified by high-performance liquid chromatography (HPLC), and subsequently linked by S-S bond via two additional terminal cysteines at the C and N termini coupled during synthesis (Neosystem, Strassbourg, France). The S-S-linked peptides were once more purified by HPLC to achieve ± 90% purity, dried, and stored at --20^{0}C until use.

ELISA. Ninety-six-well ELISA plates (Greiner Labortechniek, Germany) were coated with EBNA1 and VCA-p18 peptides using 135 ml single peptide (1 mg/ml) or combined peptides (1 mg/ml EBNA1 plus 0.5 mg/ml VCA-p18) in 0.05 M Na2CO3, pH 9.6 (Merck, Darmstadt, Germany). After overnight incubation at 4^{0}C, antigen was discarded and 200 ml blocking buffer (3% bovine serum albumin [BSA; Roche Diagnostic GmBH, Germany] in phosphate-buffered saline [PBS]) was added to each well. After a 1-h incubation at 37^{0}C, the wells were emptied and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Subsequently, 100 ml 1:100-diluted serum was applied (serum dilutions in PBS-T, 1% BSA, 0.1% Triton X-100) and incubated for 1 h at 37^{0}C. All sera were tested in duplicate. After four washings with PBS-T, rabbit anti-human IgA horseradish peroxidase (HRP) conjugate (diluted 1:4,000 in serum dilution buffer) or anti human IgG-HRP conjugate (diluted 1:6,000 in serum dilution buffer) (Dako) was added and incubated for 1 h at 37^{0}C. After four washes with PBS-T, 100 ml/well of 5-5-,3-3-tetramethylbenzidine substrate solution (bioMerieux, Boxtel, The Netherlands) was added and kept in the dark for 30 min (for IgA detection) or 10 min (for IgG detection). The reaction was stopped by adding 100 ml of 1 M H_2SO_4 (Merck, Schuchardt, Germany). The optical density was determined at 450 nm (OD450) using an ELISA reader (2001; Anthos, Austria). All OD450 values were normalized by subtracting the value for

1:100-diluted EBV-negative sera used in duplicate in each ELISA run. The receiver operating characteristic (ROC) curve was drawn to determine the cutoff values (CoV) for EBNA1, VCA-p18, and EBNA1 plus VCA-p18 (EBNA1+VCA-p18) by using a large panel of Indonesian healthy subjects (n= 254) and NPC patients (n=151). CoV were applied to determine diagnostic specificity and sensitivity (7). In initial studies prior to IgA ELISA analysis sera were pretreated with GullSorb (Meridian, The Netherlands) as described by the manufacturer in order to remove possibly competing IgG antibodies.

Immunoblot detection. Immunoblot strips containing HH514.c16 nuclear antigen induced chemically to produce the late lytic phase of EBV proteins were used to detect IgG antibody to EBV proteins. The strips were prepared and analyzed exactly as described by Middeldorp and Herbrink (30). To determine the position of characteristic EBV antigens, blot strips were incubated with monoclonal or polyclonal antibodies of known specificity and human reference sera and the EBV-specific antibody diversity profiles of IgG and IgA responses were used as confirmation of the IgA ELISA as recently described (13, 23).

Statistical analysis. All statistical analysis was done by the GraphPad Prism, ver. 4.0, program. ROC analysis was done to determine CoV; one-way analysis of variance was used to compare mean values of different antigens in IgA ELISA and differences in IgA ELISA values in relation to tumor size, lymph node involvement, and metastasis (TNM) stage, age, and sex group; and linear regression analysis was used to determine the use of Gullsorb.

RESULTS

EBV peptide ELISA. In a first series of experiment conditions for peptide antigen coating, serum and conjugate dilution were optimized (data not shown), leading to the protocol described in Materials and Methods. Optimal detection of IgA antibodies to EBNA1 and VCA-p18 separately required coating at 1 µg/ml. For simultaneous detection of IgA antibodies to EBNA1+VCA-p18 peptides, prior incubation with 1 µg/ml EBNA1 peptide for 2 h at 37^{0}C, followed by subsequent 0.5 ml/ml VCA-p18 peptide overnight proved optimal for preparing the solid phase. Best coating was obtained by using standard 0.05 M $NaHCO_3$ buffer at pH 9.6. Following peptide coating and postcoat blocking with 3% BSA, the coated plates could be dried, sealed, and stored at 4°C without significant loss of reactivity for several months (data not shown). However, in most experiments shown here weekly freshly coated ELISA plates were used.

Effect of IgG removal on IgA antibody reactivity. Because IgG reactivity to EBNA1 or VCA-p18 is present in virtually all sera and might bind identical epitopes as IgA, we compared serum reactivities with and without prior IgG removal using GullSorb, previously shown to be highly effective in removing IgG prior to IgM detection in ELISA (44). Results shown in Fig. 1 reveal that prior IgG removal did not affect the IgA reactivity of NPC sera in EBNA1 or VCA-p18 or combined EBNA1+VCA-p18 ELISA.

Comparison of IgG and IgA ELISA. Using a limited but random selection of serum specimens from each group (healthy subjects, control patients, and NPC patients), we first optimized ELISA conditions for discriminating NPC cases and controls by IgG and IgA peptide-based ELISA. Results showed 100% positive IgG antibody reactivity for EBNA1 and VCA-p18 and for the combination ELISA. The individual OD_{450} values in the combined EBNA1 + VCA-p18 IgG ELISA were always higher than for the single antigen IgG ELISA. The mean IgG reactivities from the EBNA1 + VCA-p18 ELISA for healthy carriers, non-NPC patient controls, and NPC patients from Indonesia were reflected in OD_{450} values of 2.43, 1.86, and 3.05, respectively, being significantly higher for NPC patients compared to both control populations ($P < 0.05$). However, the

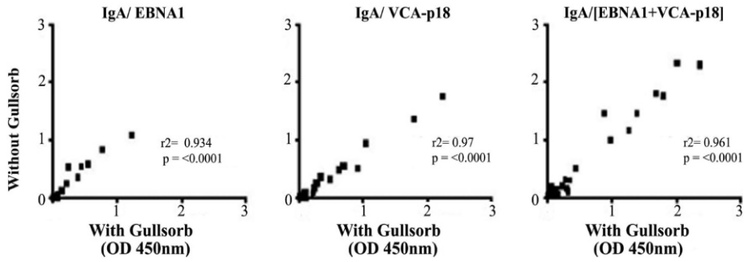

Fig. 1. The effect of GullSorb in EBV peptide IgA ELISA. Twenty-two distinct sera from confirmed NPC cases having variable IgG and IgA reactivities to EBV antigens, as defined in a previous study (13), were tested in three separate EBV peptide ELISAs for IgA reactivity either with or without prior GullSorb treatment using the protocol prescribed by the manufacturer. Correlation coefficients (r^2) > 0.9 for IgA ELISA results show that prior removal of IgG is not required, indicating that EBV-specific IgG and IgA antibodies in NPC sera are not competing and appear to recognize different epitopes. All assays were done in parallel, and identical results were obtained in repeated experiments.

wide overlap in EBV IgG ELISA reactivity between healthy carriers and non-NPC tumor controls versus NPC patients prevented its diagnostic use. For IgA ELISA the difference was more significant ($P < 0.0001$), particularly in the EBNA1 and EBNA1+VCA-p18 combination assays, the latter producing mean Od_{450} values of 0.2, 0.3, and 1.33 for healthy carriers, control patients, and NPC patients, respectively.

EBV IgA ELISA of the large panel. For the further analysis of EBV IgA reactivity we subsequently screened a larger population of healthy carriers from Indonesia (n=254) and NPC patients (n=151) collected over a 3-year period and also included several non-NPC cancer controls (n=43). The mean OD_{450} values for healthy carriers, controls, and NPC patients were 0.05, 0.20, and 0.53, respectively, for EBNA1; 0.17, 0.55, and 0.86, respectively, for VCA-p18; and 0.22, 0.24, and 1.52, respectively, for combination antigens. The mean OD450 values for NPC sera were significantly higher in all three IgA ELISAs, with the best discriminatory value for the EBNA1 + VCA-p18 combination assay ($P < 0.0001$). Results for individual samples from the larger Indonesian panel (Fig. 2) showed that in some non-NPC tumor control cases VCA-p18 ELISA detection yielded high IgA reactivity, in contrast to the EBNA1 ELISA. However, EBV IgA combination ELISA picked

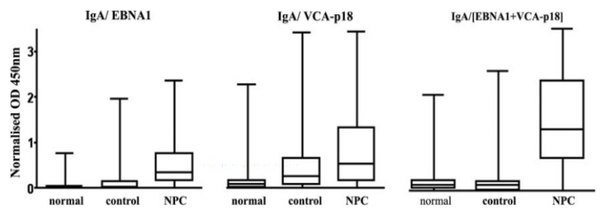

Fig 2. Distribution frequency of EBV IgA ELISA values in the complete Indonesia panel for EBNA1, VCA-p18, and combination antigen. Shown are the mean OD450 values and 25th and 75th percentiles of OD_{450} values for IgA reactivity in three EBV synthetic peptide ELISAs using Indonesian serum panels consisting of healthy blood donors (n=254), cancer control cases (n=43), and NPC patients (n=151). The results reveal that combined EBNA1+VCA-p18 IgA ELISA provides better discrimination of NPC patients and controls compared to either single antigen IgA ELISA ($P < 0.0001$).

up both signals, leading to reduced overall reactivity but with a significantly improved discrimination between NPC patients and controls ($P < 0.0001$).

In order to develop a reproducible screening assay, we defined the optimal cutoff value by ROC analysis to give the best discrimination between healthy carriers and NPC patients (8). Figure 3 shows ROC analysis of the Indonesian panel of NPC patients (n=151) and healthy regional donors (n=254) used to calculate the optimal CoV for IgA ELISA using either EBNA1 or VCA-p18 or the EBNA1_VCA-p18 combination as the solid-phase antigen. Table 1 shows corresponding CoVs and associated sensitivity and specificity values of IgA ELISAs with single and combination antigens. These results yielded PPV/NPV values exceeding 80% for EBNA1, VCA-p18, and the combination assay, respectively, as shown in Table 2. Based on the optimum CoV defined by ROC analysis, 37 (14.6%) healthy carriers and 7 (16.3%) control patients showed values above CoV in the combination assay (included two nasal cavity cancer cases, one larynx cancer case, one non-

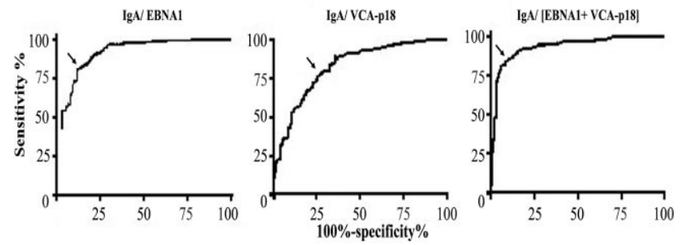

Fig. 3. ROC curve of IgA ELISA using EBNA1 and VCA-p18 peptide as single and combination antigen. The ROC curve plot was made by using the data from a large panel of sera from healthy blood donors (n=254) and NPC patients (n=151) of Indonesian origin. The ROC-determined sensitivity and specificity of the ELISA method yielded PPV and NPV values as detailed in Tables 1 and 2. Arrows show the sensitivity and specificity values chosen for each ELISA system.

Hodgkin lymphoma case, and three breast cancer cases), while 14 (9.5%) NPC cases showed values below CoV. These individuals were further analyzed by immunoblotting as described below.

NPC patients were further classified according to stage of disease, sex, age, and TNM status and analyzed for their serological response in the EBV IgA ELISA. Most patients in the Indonesia panel presented with advanced disease (87.4%), and there was a 2.63 male/female ratio, with an age distribution of 25 to 80 years (mean, 47.6 years). Our analysis showed no significant correlation ($P < 0.05$) between any of the EBV IgA ELISA values and any of the parameters tested (data not shown). Although the highest EBV IgA responses were observed in the patient with the most advanced stage of disease, the mean and median values were not statistically different from patients with early-stage disease (Fig. 4).

Comparison of IgA ELISAs for Indonesian and Chinese populations. A separate study using identical EBV ELISA conditions was also done in China, using locally collected samples from NPC patients and regional Chinese controls from the Wuzhou City region (10, 50). For this purpose, precoated, dried, and sealed ELISA plates were provided to our Chinese collaborators, together with standard serum diluents and detection reagents in concentrated form plus a work sheet. Overall results for the Indonesia and China panels are shown in Table 2, which revealed that in Chinese individuals the use of single-antigen ELISA gave a similar positive detection rate for both EBNA1 and VCA-p18 IgA as found in the Indonesia panel, whereas the use of the VCA-p18 peptide

Table 1. Cut-off values and diagnostic performance characteristics of EBV IgA ELISA in the Indonesia panel consisting of sera from healthy donors (n= 254) and NPC patients (n=151)

	EBNA1	VCA-p18	[EBNA1+VCA-p18]
Cut-off value	0.1205	0.2233	0.3536
Sensitivity (%)	88.6	79.8	85.4
Specificity (%)	80.1	70.9	90.1

was less sensitive than EBNA1. Highly comparable results were found between both Chinese and Indonesian populations in the single- test EBNA1+VCA-p18 combination ELISA, which illustrates the diagnostic potential of this single-test option for NPC diagnosis and screening in both populations.

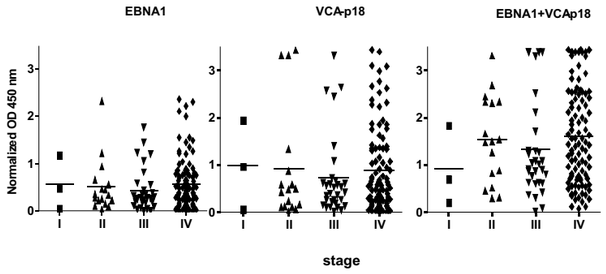

Fig. 4. Data analysis of IgA ELISA by using EBNA1 and VCA-p18 as single and combination antigens in NPC patients grouped by stage. IgA ELISA results for pretreatment sera of Indonesian NPC patients having stage I (n= 3), II (n=17), III (n=31), and IV (n=100) disease at first presentation are shown. Although peak IgA EBV reactivities increase with stage, the mean OD450 values were not significantly different between different stages (P < 0.05).

Immunoblot confirmation of discrepant samples. In order to further define the serological status of control samples with mostly weak OD_{450} values in IgA ELISA but above the CoV (37/252 healthy donors and 7/43 non-NPC patient controls) or below the CoV (for 14/151 NPC patients), the EBV IgG diversity pattern was determined by immunoblot analysis, recently shown by us to provide excellent discrimination of NPC and non-NPC patients from different geographical backgrounds (13). Figure 5A and B show IgG immunoblot patterns of 12 representative healthy controls and all 7 non-NPC patient controls, respectively. Thirty-four of 37 healthy carriers and 5 of 7 non-NPC tumor control patients with positive EBV-IgA ELISA result showed a restricted IgG diversity pattern characteristic for healthy EBV carriers. These individuals had IgG reactivity only with EBNA1 (BKRF1; 72 kDa), VCA-p40 (BdRF1, 40 to 42 kDa), and VCA-p18 (BFRF3; 18 kDa), whereas one showed an additional weak reactivity to the ZEBRA protein (BZLF1; 36 and 38 kDa) (Fig. 5A, lane 3). Two of the non-NPC tumor patient controls showed increased IgG reactivity with the major EAd marker BMRF1 (47 to 54 kDa), as shown in Fig. 5B, lanes 5 and 7, suggestive of reactivating EBV infection. These data reveal that (weakly) increased EBV IgA ELISA reactivity in control EBV carrier populations is rarely associated with increased IgG diversity, confirming our previous studies.

On the other hand, 14 of 151 NPC samples (9.3%) showed IgA ELISA values below the CoV. Ten of these (71%) showed a complex IgG recognition pattern, previously established as the “NPC pattern” (collectively represented by lane 5 in Fig. 5C). Such an NPC pattern includes additional IgG reactivities to EBV EA proteins such as EA-p138 (BALF2; 138 kDa), thymidine

Table 2. Detection of antibodies by IgA EBNA1 ELISA, VCA-p18 ELISA, and the combination of the two in both Indonesia and China panels

A. Indonesia Panel

		IgA/ EBNA1				IgA/VCA -p18				IgA/EBNA1+VCA - p18			
sera	number	(+) number	(+) rate (%)	PPV	NPV(%)	(+) number	(+) rate (%)	PPV	NPV(%)	(+) number	(+) rate (%)	PPV	NPV(%)
NPC patients	151	121	80.1	80.6	88.4	107	70.9	67.7	82.2	137	90.7	78.7	93.9
Healthy individual	254	29	11.4			51	20.08			37	14.6		
control	43	11	25.5			23	53.49			7	16.3		

B. China Panel

		IgA/ EBNA1				IgA/VCA -p18				IgA/ EBNA1+ VCA - p18			
sera	number	(+) number	(+) rate (%)	PPV	NPV(%)	(+) number	(+) rate (%)	PPV	NPV(%)	number	(+) number	(+) rate (%)	
NPC patients	145	122	84.1	91.7	81.6	125	86.2	93.3	83.9	183	174	95.1	95.6	89.3
Healthy individual	113	11	9.7			9	8.0			83	8	9.6		

kinase (BXLF1; 65 kDa), DNase (BGLF5; 55 and 58 kDa), and major-EA(D) (BMRF1; 47 to 54 kDa). Only 2/151 (1.3%) showed reactivity similar to most healthy donors and non-NPC controls (Fig. 5C, lanes 1 and 2). An additional two NPC patients showed restricted diversity but with increased intensity (Fig. 5C, lanes 3 and 4). Interestingly, immunoblotting revealed that three patients (Fig. 5C, lanes 1, 2, and 4) had no/low IgG response to EBNA1 and two patients (Fig. 5C, lanes 1 and 3) had no/low IgG responses to VCA-p18. These four sera also lacked IgA immunoblot reactivity to both markers (data not shown).

By including IgG immunoblotting as a confirmation assay for samples with EBV IgA values above the CoV, the sensitivity and specificity of EBV IgA ELISA for identification of true NPC cases increased from 85.4% to 98.6% and from 90.1% to 99.6%, respectively, and PPV/NPV values increased from 78.7/ 93.9% to 98/99.2%.

EBV IgA ELISA in acute EBV infection. In order to further define the clinical specificity of EBV peptide-based IgA ELISA, we analyzed sera from patients with acute or chronic EBV infection, which may share some of the symptoms associating with early-stage NPC disease. EBV IgA ELISA using the combination of EBNA1+VCA-p18 peptides was therefore used on a panel of sera from patients with IM and C-EBV of European origin. Figure 6 shows that none of the sera from IM patients (n= 39) showed significant IgA reactivity to the combined EBNA1+VCA-p18 antigens,

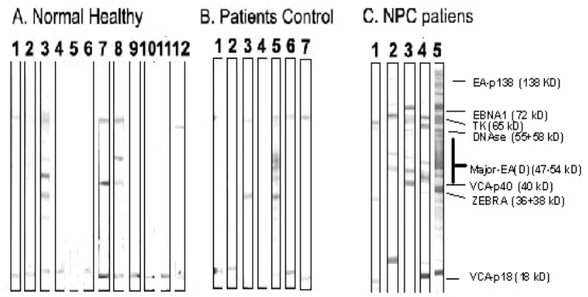

Fig 5. IgG EBV immunoblot analysis detection for confirmation of false-positive and negative results in IgA EBNA1+VCA-p18 ELISA. Three of 37 healthy subjects and 2 of 7 patient controls with high OD450 showed slightly "abnormal patterns," while 2 of 14 NPC samples with low OD450 values showed "normal patterns" (for details see the text)

and only 3 of 10 (30%) C-EBV patients showed a positive IgA result. These results support the use of single-well IgA EBNA1-VCA-p18 ELISA for the specific diagnosis of NPC.

DISCUSSION

A novel one-step EBV IgA ELISA was developed by employing two synthetic peptides derived from EBNA1 and VCA-p18 antigens for the serodiagnosis of NPC in high-risk populations in Southeast Asia. This study extends a recent immunoblot study (13), which explored the molecular diversity of anti-EBV IgG and IgA responses in NPC patients from different geographical backgrounds. In that study we noticed distinct differences in the antigen fine specificity of IgG and IgA antibody responses, suggesting independent antigen triggering of B cells. In addition, significantly different antigen recognition profiles were revealed for both IgG and IgA antibody responses between NPC patients and regional controls, permitting diagnostic use (13, 23). Immunodominant proteins were defined, including VCA-p18 and EBNA1, and reactive epitopes

were identified using PEPSCAN technology (31, 32, 43, 44). In this study we describe in detail the IgA responses against two synthetic multiepitope peptides previously proven of value for EBV-specific IgM- and IgG-based diagnosis of acute primary EBV infection (28, 32, 44).

Studies on NPC serodiagnosis have been described extensively in recent years. For example Hsu et al. (21) employed a recombinant EA and EBNA1 IgA ELISA for NPC diagnosis and screening in Taiwan and showed sensitivity, specificity, and accuracy of 98.1, 81.8, and 88.7%, respectively, for diagnosis of NPC patients and showed that the IgA ELISA values correlated with stage of disease. A more comprehensive ELISA study was done by Dardari et al. (9) with a panel of Moroccan NPC patients; this study employed multiple ELISA tests for detecting IgG and IgA response to VCA-p23-p18, EA-p54-p138, and EBNA1, in comparison to IFA IgG and IgA to VCA and EA. Their results showed that IgA EAd-p54-p138 ELISA had better diagnostic value for NPC detection (70%), compared with IgA VCA-p18-p23 and IgA EBNA1 ELISA, which particularly had limited diagnostic value in young patients. Furthermore IgA EAd-p54-p138 ELISA could detect 64% of NPC cases negative by classic IFA. The combination of IgG ZEBRA IFA with IgA EAd-p54-p138 ELISA improved the sensitivity of detection of NPC to 95% in the overall NPC population. Similarly, Chen et al. (4) generated an EBNA1 recombinant derived from an NPC biopsy and developed an IgA EBNA1 ELISA with a 78.7% positivity rate for NPC samples, which could be raised to 92.5% when combined with IgA VCA using IFA. More recently, Karray et al. (23), using identical EBV synthetic peptides as in this study, demonstrated excellent correlation between individual EBV peptide-based ELISAs and classical IFA-based serology and showed that IgA VCA p18 peptide

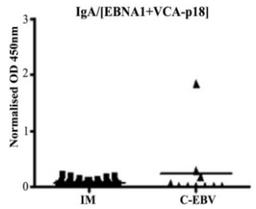

Fig. 6. IgA EBNA1+VCA-p18 ELISA of acute EBV infections. EBNA1+VCA-p18 combination peptide IgA ELISA was used for analyzing EBV IgA reactivity in sera from patients with acute IM (n = 39) or C-EBV (n = 10). Data show the absence of significant EBV IgA reactivity in IM patients and variable reactivity in about 30% of CEBV patients in this assay.

ELISA raised the detection rate from 78.6% to 89.8% in young Tunisian NPC patients compared to IFA. These studies, however, all showed that a single serological test cannot achieve the ideal objective of identifying all NPC patients, and it was advised to combine different tests for diagnosis of NPC. Combinations of different technologies, how-ever, add to cost and may be difficult to realize in developing countries. A single-assay format is preferred. Peptides may substitute for natural or recombinant proteins as a stable, reproducible, and cheap source of antigen in ELISA. VCA-p18, the small capsid protein of 18 kDa encoded by the BFRF3 gene, is highly immunogenic in humans, and its use in ELISA is well described (3, 9, 30, 35, 44). The EBNA1 synthetic peptide used in this study derived from a unique region of EBNA1 (combining amino acids 382 to 410 and 413 to 452) not including glycine-alanine repeat sequences, which may produce nonspecific reactivity (27, 32).

Compared to the studies mentioned above, we here show that a synthetic-peptide-based EBV IgA ELISA, combining VCA-p18 and EBNA1 epitopes into a single ELISA well, can achieve sensitivity and specificity values above 85% in two independent NPC populations from Indonesia and southern China. Importantly, this assay not only allows discrimination between NPC patients and healthy EBV carriers but also between NPC and non-NPC tumor patients in regions with high

NPC prevalence. Importantly, we show that the EBV IgA EBNA1+VCA-p18 combination peptide ELISA permits discrimination of NPC patients from patients with acute or chronic active EBV infection, which may present with similar nonspecific symptoms. This is relevant for diagnostic screening in populations with symptoms in head and neck suspicious of NPC. Therefore this well-defined assay may be suitable for large-scale diagnostic screening in high-risk regions. However, we failed to find a direct correlation between EBV IgA reactivity and TNM stage, as was found in another study (21). This might be related to the relatively high sensitivity of the EBNA1 + VCAp18 peptide combination ELISA for detecting early stage NPC, but this remains to be proven in more-extended studies, because early-stage samples were rare in our population.

Our data reveal that removal of IgG by using Gullsorb is not required for optimal EBV IgA detection by ELISA, indicating that IgG and IgA may react with different epitopes or that IgA avidity is sufficient to compete with IgG. This is in line with our previous data showing that IgG and IgA responses have distinct EBV antigen specificities and may be triggered differently (13). We show by comparing the IgG and IgA ELISA results for EBNA1 and VCA-p18 peptide antigens either alone or in combination that the IgG levels in all three populations were higher than IgA, being highest in NPC patients ($P < 0.05$), with 100% of tested individuals having a positive result in the EBNA1 + VCA-p18 combination test. The 100% EBV IgG positive rate is not surprising, since in Asia most individuals acquire EBV during early childhood (20, 48). However, the considerable overlap in OD_{450} values in EBV IgG combination peptide ELISA between healthy donors and NPC patients precludes its diagnostic use in the NPC setting.

On the other hand, mean EBV IgA antibody levels in the NPC panel were significantly higher ($P < 0.0001$) that those of healthy blood donors and control patients, either in single- or double-antigen ELISA (Fig. 3). Sensitivity of combined EBNA1 + VCA-p18 peptide ELISA (85.3%) was lower than EBNA1-only ELISA (88.5%), but it showed higher specificity (90.1%) compared to either EBNA1 or VCA-p18 as a single antigen. The relatively high reactivity of IgA VCA-p18 in Indonesian non-NPC patients may be explained by (subclinical) EBV reactivation and local EBV replication in the nasopharynx (51) due to poor health conditions, similar to recent observations by us for human immunodeficiency virus carriers (39). EBNA1 IgA responses appear to be more specific for NPC and may be related to release of EBNA1-DNA complexes from dying NPC tumor cells (40). However, not all NPC patients have a detectable EBNA1 IgA response, and combination with VCA-p18 in an IgA ELISA clearly improves diagnostic use.

In order to increase the specificity and sensitivity of serological diagnosis of NPC, we employed a “second-line” IgG immunoblot test as a confirmatory assay. In NPC patients, IgG antibodies typically react with a broad range of EBV lytic proteins (13). By combining EBV IgA ELISA with IgG immunoblot analysis of samples showing an initial positive ELISA result, specificity/sensitivity and PPV/NPV for NPC detection increased to > 95%. Using the current EBNA1 + VCA-p18 peptide mixture some 9% of NPC patients would be missed in an IgA ELISA-based screening program, and ideally these patients should be identified. Immunoblot analysis revealed 4/14 (29%) of these nonresponders indeed had low-to-undetectable EBNA1 of VCA-p18 IgG and IgA responses, confirming the ELISA results. However, additional IgA-reactive bands were identified for most of these sera, which are mainly characterized as EA complex proteins (Fig. 5C). Further work is in progress to define additional IgA-reactive proteins and epitopes and to further increase the overall sensitivity of the synthetic-peptide ELISA approach for primary NPC screening in high-risk populations.

Overall, the combined IgA EBNA1+VCA-p18 ELISA showed promise as a tool for primary diagnosis of NPC in Indonesian and Chinese populations compared to a single antigen IgA ELISA. It provides an important step to development of a well-standardized and affordable test for use in

high-risk populations. The lack of significant IgA responses to EBNA1 and VCA-p18 combination peptides in acute and chronic EBV infection further supports the potential use of EBV IgA combination peptide ELISA as an NPC-specific screening tool. The inclusion of a confirmation assay is advocated for individuals with a positive result in the initial IgA ELISA screening. The immunoblot technique or EBV DNA load analysis may well fulfill this purpose (2, 13, 23, 24, 26). Importantly, it was recently shown that EBV DNA load measurement may provide an independent marker for NPC, not related to serology (24, 40). A combined positive result in independent tests may be used to select individuals for detailed clinical examination including endoscopy and biopsy. Finally, it should be stressed that previous large-scale seroepidemiological studies in China and Taiwan revealed that apparently healthy individuals with elevated EBV IgA responses have significantly increased risk for NPC development in subsequent years (6, 10). Such individuals may also be included among the healthy donors with positive EBV IgA ELISA result identified here. However, this awaits clinical confirmation in more detailed follow-up studies.

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CHAPTER 4

Combination of Epstein-Barr virus scaffold (BdRF1/ VCA-p40) and small capsid protein (BFRF3/ VCA-p18) into a single molecule for improved serodiagnosis of acute and malignant EBV-driven disease

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ABSTRACT

Current single molecule EBV markers fail to reach 100% sensitivity in serodiagnosis of acute and malignant disease. A previous study (van Grunsven et al., 1994) had identified immunodominant epitopes of VCA-p40 and VCA-p18, and indicated that those two VCA antigens may have diagnostic value for EBV-related diseases. We now have developed a recombinant protein of full length BdRF1 fused to the immunodominant domain of BFRF3 as 6-his tagged protein in *E. coli*. The recombinant protein was extracted in 8M urea solution and purified by metal-affinity chromatography yielding a 55 kD product (VCAp40+18). VCAp40+18 blot-strip tested for IgM reactivity in infectious mononucleosis (IM) samples yielding 100% sensitivity and specificity, with improved reactivity compared to IgM/VCA-p18 ELISAs.

In a recent study we described a synthetic peptide based IgA/[EBNA1+VCA-p18] ELISA, with sensitivity about 90% for diagnosing nasopharyngeal carcinoma (NPC). Immunoblot analysis of biopsy confirmed NPC cases with low of negative IgA/[EBNA1+VCA-p18]-ELISA showed 100% IgA reactivity to VCA-p40 (BdRF1 protein) and VCA-p18 (BFRF3 protein). We evaluated VCAp40+18 as additional marker for screening and diagnosis of NPC. Our data show positive IgA/VCAp40+18 reactivity in ELISA for 17/22 (80.95%) of NPC samples that were missed by peptide-based IgA/[EBNA1+VCA-p18] ELISA, suggesting VCA-p40+18 as improved marker for NPC serodiagnosis. The VCAp40-p18 may be combined with EBNA1 synthetic peptide in a mix or in separate IgA-ELISA test for improved NPC serodiagnosis.

INTRODUCTION

Epstein - Barr virus (EBV) infects almost 90% of the population worldwide. It is acquired during childhood in most cases and establishes a life-long persisting infection that is rarely symptomatic. In developed countries, primary infection occurs frequently during adolescence, which leads to infectious mononucleosis (IM) in approx. 30% of cases. IM is a benign disease with the expansion of paracortex of lymphoid tissue vigorously producing polyclonal CD8+ T cells (23). Primary EBV infection is marked by temporal IgM reactivity towards viral capsid antigen (VCA) and early antigens (EA) complex, as well as IgG to EA-related proteins, and these responses will disappear during convalescence (17, 32). Presence of IgM antibodies to VCA provides an accurate marker for diagnosis of acute IM (2). IgG reactivity to VCA develops soon after infection and will persist for life. Meanwhile IgG reactivity to EBNA1 will appear weeks or even months after EA and VCA responses (14). IgA reactvitiy towards EA (BZLF1 and BMRF1) and VCA antigens were also found in 100, and 78% of samples tested, respectively, which decreases rapidly after the acute phase of infection (1). This finding suggests that lytic viral replication takes place at mucosal sites (1, 26).

In general, EBV healthy carriers show a limited diversity of IgG antibodies directed to EBNA1, VCA-p18, VCA-p40, gp125, p160, and immediate early protein BZLF1 (11). However, in patients with EBV-related diseases IgG and IgA responses to EBV proteins show a different reactivity pattern, reflecting different antigen/ epitope triggering (11)

EBV infection is associated with several malignancies and especially the undifferentiated type of nasopharyngeal carcinoma (NPC WHO type III) shows almost 100% etiological relationship (23). NPC patients show an abnormal anti-EBV antibody profile and increased circulating EBV DNA levels (11, 16, 31). NPC patients especially exhibit high IgA antibody response to a large spectrum of EBV antigens, including EBNA1 and VCA-, and EA-related proteins (11). Elevated IgA responses to EBV antigens may precede NPC development at early stage (20).

To date, ELISA is the most convenient serological method for NPC diagnosis, being easy to perform and suitable for high-throughput applications. Selection of a suitable antigenic marker is key to develop a good ELISA system aiming for high sensitivity and specificity (Se/Sp). Previous studies have explored the serological use of various antigens from EBV latent and lytic genes, including purified native or recombinant proteins and EBV protein-derived synthetic peptides (6, 8, 9, 19, 28, 37) For NPC serodiagnosis, a single marker may not be sufficient to identify all individual NPC patients, regarding the observed diversity of antibody reactivity among individual NPC patients (11). For that reason, immunofluorescence assays (IFA) are still widely used, providing a summary result of responses to multiple individual EBV proteins and epitopes (12, 33). However, this technique is restrained by technical difficulties, subjective to result interpretation, and not suitable for high-throughput. Complementary tests of different serological markers in separate or single assays (4, 6) and the combination of antibody response and EBV DNA load were also proposed for diagnosing NPC (12, 33).

We have developed a one-step EBV-IgA ELISA based on synthetic peptides derived from immunodominant epitopes in EBNA1 and VCA-p18 (IgA/EBV-ELISA). The application of this method for NPC diagnosis showed a Se/Sp of about 90% (9) . This method is used for routine NPC diagnosis at our hospital. However, we still miss approximately 10% of biopsy confirmed NPC by using the IgA/EBV-ELISA. Therefore, we need to identify additional IgA-reactive EBV protein(s) and/or epitopes in order to improve the overall serological Se/Sp of our system.

In this article, we analysed the spectrum of antibody responses of confirmed NPC patients with low/negative IgA/EBV ELISA result in first samples, to reveal suitable additional serological markers. We defined a recombinant VCA protein marker consisting of full length BdRF1 fused with an immunodominant region of BFRF3 (35), which shows improved serological reactivity. We show preliminary results to determine its diagnostic relevance for acute IM and NPC serodiagnosis.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 130 Indonesian patients with histologically-proven NPC. EBV positivity of the tumours was confirmed by EBER-RNA in situ hybridization and immunohistochemical staining for EBNA1 (3, 10). Sera from healthy blood donors (n=117) were obtained from the local Red Cross blood bank in Yogyakarta. Sera from acute infectious mononucleosis (IM) cases (n=12) were recently described (29). Sera from healthy donors of known EBV status served as controls (reference sera). IM serology was confirmed by positive VCA-IgM and high IgG/ EA(D) values and low or negative IgG/ EBNA1 values as described before or by commercial RecombLine test (22).

Monoclonal/ polyclonal antibodies. Rabbit anti-DNAse (K120), mouse anti-DNAse (OT18C), mouse anti-ZEBRA (BZ1), rat anti-VCA-p18 (OT15E), rabbit anti-VCA-p40 (K8) (11), and mouse anti-histidine tag (Bionova GmBh Hamburg, Germany) were used to confirm reactivity of purified recombinant DNAse, ZEBRA, and VCAp40+18 proteins, respectively, as well as being used as positive control in developing IgA-ELISA. All monoclonal/polyclonal antibodies were stored in -20°C until used, unless otherwise recommended.

Immunoblot detection. Immunoblot strips containing HH514.c16 nuclear antigen induced chemically to produce the early and late lytic phase of EBV proteins were used to detect IgG antibody to EBV proteins. The strips were prepared and analyzed exactly as described previously (24). The position of characteristic EBV antigens had been identified by monoclonal and polyclonal antibodies, and comprised of VCA-p160 (BcLF1; 160kD), EA-p138 (BALF2; 138 kD), EBNA1 (BKRF1, 72 kD), thymidine kinase (BXLF1; 65kD), DNAse (BGLF5; 55+57 kD doublet),

Major-EA(D) (BMRF1; 47-54 kD-diffuse), VCA-p40 (BdRF1; 40kD), ZEBRA (BZLF1; 36+38 kD; fine doublet), and VCA-p18 (BFRF3; 18 kD) (5, 11).

EBV recombinant proteins and peptides. Recombinant DNAse type A and B (BGLF5) (DNAse type B was kindly provided by Dr. T.Ooka (Lyon, France) and ZEBRA (BZLF1) proteins were each expressed in SF9 cells, and constructed as described previously (28). Briefly, the recombinant DNAse protein was harvested from sonified SF9 cell lysate, filtrated trough 0.22 µm filter to remove cell debris, and purified in cation exchange column material (Mono-S, Amersham Pharmacia, Sweden) with elution buffer (20mM KH_2PO_4, pH8,0 20%Glycerol 0,5mM Dithiothreitol 2mM $MgCl_2$). ZEBRA recombinant protein was harvested similarly from SF9 cell lysate, and purified over Nickel charged chelating sepharose fast flow column material (Amersham Pharmacia, Sweden) in buffer containing 8 M urea, and eluted with imidazole gradient concentration (similar as VCA-p40+18 recombinant purification; see below). A peptide derived from the N-terminus of ZEBRA (OTP513) was also used, and described to represent immunodominant epitope (3). Recombinant VCA-p160 (Biosource, Nivelle-Belgium) and 6 peptides-derived from VCA-p160 (1879A, 1469A, 1395A, 1761B, E27G, and WW1469) were used as well (25). Peptides derived from immunodominant epitopes of EBNA1 and VCA-p18 were defined as described previously (9, 35). Fusion of BdRF1 full length gene (encoding VCA-p40) and BFRF3 encoding immunodominant epitope of VCA-p18 was expressed in *E.coli* as described below. All recombinant proteins and peptide-derived from EBV antigens were stored at -20^{0}C until use.

IgA-ELISAs. IgA/[EBNA1+VCA-p18] ELISA was done as described previously (9, 10), while IgA-ELISA using DNAse type A and B, ZEBRA, and VCA-p160 recombinant proteins and peptides were optimised first in an indirect ELISA system, with identical standard buffers used in IgA/[EBNA1+VCAp18]-ELISA (9). Differences were found in antigen coating, and chromogen incubation time. The antigen coating was 1:100 for DNAse and ZEBRA recombinant antigens, and 1ug/ml for VCA-p160 purified protein and peptides. While chromogen incubation time were 10, 45, and 30 minutes for DNAse, ZEBRA, and VCA-p160, respectively.

Expression, purification, and application of VCAp40+18

(1). Cloning and expression of recombinant VCAp40+18 protein in E.coli. pQE-Tri plasmid containing amp^r (Qiagen, Strausse, Germany) was used to insert full length sequence of the BdRF3 gene sequence fused to the immunodominant C-terminal epitope (peptide IV) of BFRF3 (35). A six-histidine-tag sequence was placed directly downstream the inserted proteins sequence. *E. coli* was transformed with pQE-Tri-p40-p18 and single colony was picked and cultured in LB+ 0.1% ampicilline at 37^{0}C in shaker incubator. Optimalization of VCAp40-p18 expression was done by several methods, i.e. (1). using different *E.coli* strains for plasmid transformation, E. coli DH5α, SG13009, and M15; (2). The addition of 1mM IPTG (isopropyl β-D-1-thiogalactopyranoside) to the culture at mid log phase ($_{OD600\ nm}$=0.4-0.6); and (3). Harvest time, from several hours after mid log phase or overnight culture. Optimum strain and culture condition were then used throughout to produce recombinant VCAp40-p18.

(2). Purification of recombinant protein. Briefly, cells from overnight 100ml culture were collected by centrifugation (4000rpm, 20min). The pellet was resuspended in lysis buffer (0.05M NaH_2PO_4; 0.3M NaCl; 0.05 M EDTA, 0.25% Tween-20 and 1% lysozyme; pH 7.5). The suspension was then freeze-thawed three times subsequently in liquid N_2 and hand warm water, followed by sonification approximately 500W, 6-10 times (10 seconds each). The suspension was centrifuged (16.800 rpm, 60min, 4^{0}C), and the pellet containing non-soluble inclusion bodies was then

collected.

The pellet was dissolved in 5ml buffer containing 8 M ureum, 100mM NaCl, 20mM imidazole, 1% PMSF; pH 8.2. Protease inhibitors (leupeptine, tripsin inhibitor, aprotinin, and PMSF) were added to the lysis buffer. Debris was removed by centrifugation and 0.2 µm filtration and the flow-through was added to 2 ml of Nickel-loaded agarose (Ni-NTA resin, Qiagen GmbH, Germany), and left for 60mins at RT with rotation. The resin suspension was then centrifuged (14000rpm, 15min, RT), and the pellet was washed with 4 ml washing buffer (8 M ureum, 100mM NaCl, 40mM imidazole, 1% PMSF; pH 8.2) for 30 min at RT in rotation. Washing steps were repeated three times. Then the pellet was dissolved in 500 ul of elution buffer (8 M ureum, 100mM NaCl, 100mM imidazole, 1% PMSF; pH 5.9) for 15mins at RT with rotation and centrifuged at 14000rpm for 15min. The elution step was repeated three times to ensure complete elution, with each fraction saved and stored in -20^{0}C until used. Fractions of every step were collected for analysis.

(3). Electrophoresis and Western Blot analysis of VCAp40+18 recombinant protein. To analyze the expressed protein, samples were prepared by boiling in Laemmli sample buffer. The protein samples were separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue or transferred electrophoretically to 0.2-mm nitrocellulose membranes (Schleicher & Schuell, Germany). For immunoblot analysis, the blotted membranes, used entirely or cut into 3 mm strips, were immersed for 1 h in blocking buffer (5% [vol/vol] horse serum [Gibco BRL] and 5% [wt/vol] non-fat dry milk in PBS), to prevent nonspecific binding. A 1:50 dilution of anti-BFRF3 (OT15 E, rat monoclonal), 1:50 anti-BdRF1 (K120, rabbit polyclonal), 1:100 anti-his-tag monoclonal antibodies (Dianova), or 1:100 human serum, made in blocking buffer, were applied as first antibody to separate strips for1 hr at RT, followed by three washes in PBS-T. Detection of bound antibody was done exactly as described before (11, 28). Stained strips were washed overnight with 10 ml of H_2O, dried, and stored in the dark until documented.

(4). Western blot analysis of anti VCAp40+18 antibody in NPC and IM sera. Purified VCA-p40+18 recombinant protein were run in 10% SDS gel and subsequently transferred by western blot, as mentioned above. Blotted membrane was cut into 3-mm strips, and each strip was tested with 1:100 dilution of human sera. Antibody detection was done as described previously (11). For IgG detection (NPC and healthy donor samples), rabbit anti-human IgG HRP was used, while for IgM detection (IM serum samples), rabbit anti-human IgM-HRP were used both at 1:1000 dilution (Dako, GLostrup, Sweden). Prior to IgM analysis, IM sera were treated with GullSorb (Meridian Diagnostics, The Netherlands) as described by the manufacturer in order to remove possible competing IgG antibodies (35).

(5). IgM/ VCA ELISA. VCA-p18 synthetic peptide was made and IgM/ VCA-p18 ELISA was performed as described elsewhere (van Grunsven, Spaan et al.,1994) (25). Serum samples were treated with GullSorb (Meridian Diagnostics, The Netherlands) prior to use, to remove IgG antibodies, as described by the manufacturer. Known EBV negative and positive sera were used as control in each run.

(5). IgM/ VCA-ELISA. ELISA plate (bioMerieux, Boxtel, The Netherlands) comprising an IgM capture ELISA using VCA-p18 (BFRF3) synthetic peptide (the same antigen as described in above method) as antigen combined with rat monoclonal antibody to VCA-p18 as conjugate. The assay was performed as described by the manufacturer.

(6). IgA/ VCAp40+18 ELISA. ELISA plates (Biotek, Canada) were coated with 1 ug/ml of purified p40+18 recombinant protein in 0.05 M Na_2CO_3, pH 9.6 (Merck, Darmstadt, Germany). After overnight incubation at 4^{0}C, antigens was discarded and 200 ml blocking buffer (3% bovine serum albumin [BSA; Roche Diagnostic GmBH, Germany] in phosphate-buffered saline [PBS]) was

added to each well. After one hour incubation at 37°C, the wells were emptied and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Subsequently, 100 ml 1:100-diluted serum was applied (serum dilutions in PBS-T, 1% BSA, 0.1% Triton X-100) and incubated for 1 h at 37°C. All sera were tested in duplicate. After four washings with PBS-T, rabbit anti-human IgA-horseradish peroxidase (HRP) conjugate (diluted 1:4,000 in serum dilution buffer) (Dako) was added and incubated for 1 h at 37°C. After four washes with PBS-T, 100 ml/well of 5-5-,3-3-tetramethylbenzidine substrate solution (bioMerieux, Boxtel, The Netherlands) was added and kept in the dark for 30 min. The reaction was stopped by adding 100 ml of 1 M H_2SO_4 (Merck, Schuchardt, Germany). The optical density was determined at 450 nm (OD450) using an ELISA reader (2001; Anthos, Austria). All OD450 value was normalized by subtracting the mean value of 1:100-diluted EBV-negative sera used in duplicate in each ELISA run. Cut-off value was determined by using mean OD450 values of multiple healthy controls + 2SD.

To test possible interference of *E.coli* cellular antigens in IgA-ELISA, ELISA wells were coated with appropriately diluted cell lysate of Dh5α with empty pQE-Tri plasmid, and compared the OD450 readings with the optimized IgA/VCAp40+18 ELISA.

RESULTS

IgG/ EBV immunoblot detection. We performed peptide-based IgA/ [EBNA1+VCA-p18]-ELISA to all samples (n= 562) of our NPC panel collected during year 2001-2006. Samples (n= 49; 8.72%) with negative IgA/ [EBNA1+VCA-p18]-ELISA were decoded and tissue blocks were recovered for analysis. We were able to retrieve 27 paraffine-embedded tissues; from some (11/27) no good sections could be made for re-examination, with 16/27 giving positive EBER result. From these 16 EBER positives, being negative in the initial IgA/(EBNA1+VCAp18) screening ELISA, 6 were positive in an updated test version (new brand of ELISA plate) of the same test, and all showed aberrant immunoblot profile, similar to our previous study (11). The IgG and IgA immunoblot strip detection are illustrated in fig. 1 and summarized in table1. Fig.1. showed that IgG reactivity are directed towards a broad range of EBV antigens, and much lesser in IgA reactivity. When summarized (see table 1), IgG showed to react 100% to VCA-p40 and p18 (weak IgG/ VCA-p18 responses are shown in sample no. 7 and 9) ; 70% to EBNA1, EA(D), and ZEBRA, and less to EA-p138 and TK; while IgA reactivity was only 0-30% towards above-mentioned EBV antigens.

The 10 sera remaining IgA-EBV negative, but with immunoblot positive reactivity, were further used to evaluate novel markers for EBV serology (i.c. VCAp18+40) in order to improve screening test sensitivity.

IgA-ELISAs. Purified recombinant DNAse A and B, and ZEBRA were obtained, yielding single immunoreactive bands at 57 kDa for DNAse protein (K120 antibody), and 38 kDa band for ZEBRA protein (BZ1-antibody), respectively. VCA-peptides were purified by HPLC and used in ELISA directly.

Optimization of IgA-ELISA using DNAse A and B, ZEBRA, and VCA-p160 recombinant proteins and peptides were done with NPC and normal healthy donor sera. Only weak signals were obtained in IgA/ VCA-p160 using recombinant and peptides-derived VCA-p160, whereas IgA/ DNAse showed indiscriminate signals between NPC and healthy EBV positive donor samples. Therefore we did not further explore the development of IgA/ VCA-p160 and IgA/ DNAse-ELISAs.

Optimized conditions were determined for IgA/ ZEBRA recombinant and IgA/ OTP 513. IgA/ ZEBRA showed Se/ Sp of 74.7%/ 53.8% (CoV= mean healthy donors + 2SD) when tested to NPC (n=87) and healthy individuals (n=65). Similarly IgA/ OTP513 showed Se/ Sp of 93.8%/ 42.5% (CoV = mean healthy donors + 2SD) when tested to NPC (n=40) and healthy donors (n=32). (data not shown).

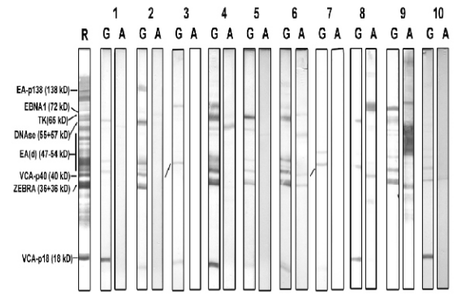

Fig.1. IgG and IgA Immunoblot analysis of NPC sera (n=10) with initial negative IgA-EBV-ELISA. Overall, samples show diverse IgG and IgA reactivity towards EBV antigens. Lane R represents a chronic EBV serum with strong immunoblot diversity. The molecular analysis of IgG and IgA responses in NPC sera is summarized in table.1.

Expression and Purification of VCAp40+18

Culture optimisation. At first, pQE-Tri inserted with full length BdRF1 gene and BFRF3 sequence encoding immunodominant epitope (named as pQEtri-p40p18 throughout this article) was cloned in *E. coli* strain Dh5α. A single colony grew in LB agar containing ampicilline (250mg/ml) was transferred to the liquid media, and grew overnight at 37°C. Supernatant (soluble part) and cell pellet (insoluble part) were sampled for analysis on 10% SDS page and western blot analysis. Fig.2. showed western blot detection by using OT15E (anti rat VCA-p18) revealing that the

Table 1. Percentage (%) of IgG/ IgA immunoblot reactivity towards EBV EBNA1 and lytic proteins in EBER-confirmed NPC sera with negative IgA/[EBNA1+VCA-p18]-ELISA result (n=10)

	p138	EBNA1	TK	Dnase	EA(D)	p40	ZEBRA	p18
IgG	30	70	40	40	70	p40	70	100
IgA	10	20	0	10	0	30	20	0

expected recombinant protein was detectable more in the cellular fraction (lane 1) compared to in the culture medium (lane 2).

We also found that the expression of recombinant protein was quite low, therefore, optimization was required. Optimization was done in three ways, first, by using different *E.coli* strains i.e. SG13009, Dh5α, and M15. The second, was by IPTG induction at mid log phase (OD_{600nm} = 0.4-0.6), and harvested three hours and overnight after induction. Fig.3B. showed western blot detection of the insoluble part of the culture with mouse anti-his antibody. SG13009 with (lane 3) and without IPTG induction (lane 7) harvested after 3 hours of culture, showed a comparable protein expression compared to an overnight Dh5α culture without IPTG induction, from previous experiment (lane 1), while M15 showed negative results with (lane 5) and without (lane 9) IPTG induction.

Dh5α was then re-tested for IPTG induction and harvesting time. Analysis is shown in fig.4. Mouse anti-histidine antibody detection (1:100) showed that IPTG induction did not increase level of protein expression, at overnight culture (Fig 4B lane 5,6) and even less at 3 hours of culturing (Fig.4B lane 7,8), compared to DH5α with overnight culture, without IPTG induction, from

previous experiment (Fig 4B lane 9).
In the end, these attempts to optimize protein expression levels did not give better protein yields, and it was decided to use an overnight Dh5α without IPTG induction to produce VCAp40+18 for further experiments.

Purification of recombinant protein. Fig.5 shows samples of every step of purification as CBB staining of 10% SDS-PAGE (fig 5A) and immunoblot detection with seropositive serum (JM), anti VCA-p18 monoclonal antibody (OT15E), rabbit anti VCA-p40 (v8), or anti his-tag (fig.5B). All show the same recognition at the same protein band, 55kD, later named VCAp40+18.

Repeated freeze-thawing and two times sonification were adequate to release the

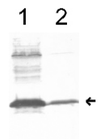

Fig.2. Western blot analysis of cellular fraction (1) and culture media (lane 2) recombinant DH5α transfected with pQE-Tri-VCAp40p18. Detection of recombinant protein was done with OT15E 1:50 (rat anti VCA-p18 antibody), showing the intended VCAp40+18 recombinant protein at about 55kDa (see arrow) both in the cellular and culture media fractions, Most recombinant VCAp40+18 protein was associated with the cellular fraction.

VCAp40-p18 recombinant protein from the cells, as shown in lane 2, where VCAp40+p18 proteins is already detected in the supernatant after first sonification. Two times washing steps were sufficient to release most non-bound proteins (lane 5,6), but still some VCAp40+18 is washed-away. At the first elution step (lane 7), a single protein band was obtained (at position about 55 kDa) at high purity compared to elution fraction 2 and 3 (lane 8,9) with only trace of a cellular protein doublet at about 35+37 kDa of which the intensity reduced in the next elution fractions (lane 8,9). Addition of plasmid-free Dh5α cell lysate to purified VCAp40+18 in the IgA/ VCAp40+18-ELISA showed no difference of $OD450_{nm}$ value with those of without additional of plasmid-free Dh5α cell

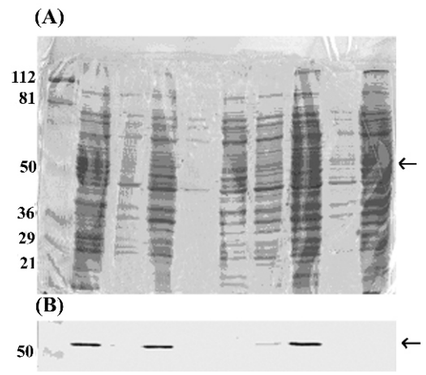

Fig.3. Insoluble fraction of VCAp40+18 recombinant protein (by coomassie stain (A) and immunoblot with mouse anti his-tag 1:100 (B). Optimization comprised of using of different *E. coli* strains , i.e. DH5α (lane 2), SG13009 lane (3,4,7,8) and M15 (lane 5,6,9,10), with IPTG induction at the initial (t=0 hour) (lane 3,5) and log phase (lane 4,8) and without IPTG at the initial (t=0 hour) (lane 5,9) and log phase (lane 6,10). Result showed that VCAp40+18 could be expressed in DH5á and SG13009, and not in M15. IPTG induction did not increase p40+18 expression (1: protein molecular weight marker (kDa); arrow : VCAp40+18 recombinant protein)

lysate (Fig.6). This may indicate that IgA to *E.coli* cellular protein was not present in the samples tested and the faint extra band showed in the VCAp40+18 eluate may not create a problem. The protein product (Fig. 5. eluate 1,2= lane 7,8) was further used in blot strip detection in acute IM and NPC, as well as ELISA development for NPC.

Additional steps, such passing cell pellet+ loading buffer trough 0.22 µm filter and the

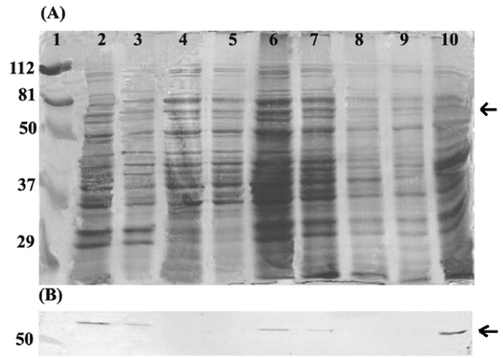

Fig.4. VCAp40+18 recombinant protein in Dh5α by coomassie stain (A) and western blot detection by using mouse anti His-tag monoclonal 1:100 (B). Optimalization was comprised of culturing with IPTG induction at log phase (8,9) and overnight (6,7) and overnight culture without IPTG induction (10). Result showed that IPTG induction and culture harvesting at the log phase did not increase the expression of VCA-p40+18 recombinant protein (1: molecular weight marker (kDa); arrow : recombinant VCAp40+18 protein)

additional of protease inhibitors afterwards, shows to reduce cellular contaminant during purification steps, as shown in fig. 7B, where two washing steps have already cleared proteins and the VCAp40+p18 (lane 8,9), plus its eluates contain only a single VCAp40+18 protein band (lane 10,11,12), using IgGof a reference healthy seropositive individual (JM). However, overall experiment shows that the loading efficiency was low, as shown in comparison of pre (lane 7) and post (lane 8) loading material where VCAp40+18 passed trough (unbound to the column matrix), with consequent low yield of VCAp40+18 purified protein (very low intensity on VCAp40+18 protein band with CBB staining at fig 7A lane 10-12). Increasing column volume and incubation time may solve this problem.

IgM/ EBV detections of acute IM patients. Table 2 shows the results of several serology tests with the acute IM panel. IgM/VCA-p40+18 immunoblot reactivity was detected in all IM samples tested, compared to 60% and 78% of in house (indirect system) and commercial (capture system) IgM/VCA-ELISA respectively. The VCA-p18 antigen used in the ELISAs is the same as used elsewhere. (9, 35). Our VCAp40+18 antigen contains the same VCA-p18 amino acid sequence, suggesting that the increased IgM-reactivity is contributed by epitopes in the VCA-p40 part of the fusion protein.

IgA/ VCA-p40+18-ELISA. Fourty-two NPC samples with negative (n=22) (“low NPC”) and positive (n= 20) (“high NPC”) IgA/[EBNA1+VCA-18]-ELISA result (fig.8) were tested with IgA/ VCAp40p18 ELISA and compared to sera from healthy subject (n=12). Minimal, median, and maximal value of low NPC panel was 657; 3,969.5 ; and 27,904.5; respectively, 0; 23,071; 27,904.5 for “high NPC” respectively and 4; 1,024.8; and 2.048 respectively for healthy subjects. The mean OD450value was found to differ significantly between non/low and high responders in

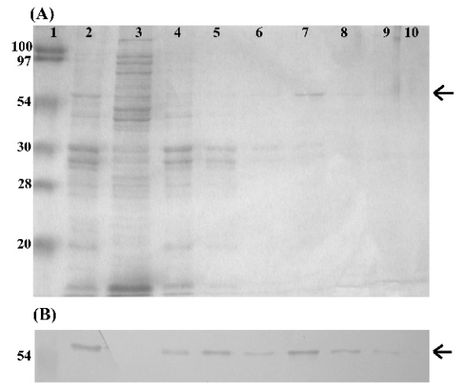

Fig.5. Flow-through of VCAp40+18 protein purification steps run by coomassie staining (A). The same flow-trough run was then detected by monoclonal anti VCA-p40 (v8; 1:50), anti VCA-p18 (OT15E; 1:50), and anti his-tag (1:100), recognizing the same protein band at position about 55kDa (B). Purified VCAp40+18 at step no. 7+8 was then used throughout the study. (1. molecular weight marker (kDa); 2,3. sonications; 4.loading material; 5,6.washings; 7,8,9 eluates; 10 column material after purification)

IgA/(VCAp18+EBNA1)-ELISA ($p< 0.05$), as may be expected on the basis opf high VCA-p18 IgA reactivity in the latter group (9). Of the 22 NPC cases missed in the screening test (IgA/(VCAp18+EBNA1)-ELISA), 13 gave a positive response and additional 1 gave borderline response in IgA to the VCAp18+p40 fusion protein. Cut-off value for IgA-VCAp18+p40 ELISA was determined as mean+2SD of OD450 value in healthy controls (CoV: 2,351) resulting 14 of 22 (63.6%) of low NPC and 19 of 20 (95%) of high NPC were above CoV, and none of normal healthy subjects (0%) tested. These data indicate that the VCAp18+p40 may have added value for IgA-ELISA in diagnosing NPC cases.

DISCUSSION

IgA response to EBV antigens is a hallmark of NPC (15), permitting the development of EBV-based serology tests for NPC diagnosis. ELISA may replace IFA for its technical simplicity, relatively low cost, (semi-) quantitative result, and objective interpretation. However, for ELISA defined and purified EBV specific marker(s) is (are) required. Our prior studies aiming to develop a diagnostic tool using the synthetic peptide-based IgA/[EBNA1+VCA-p18]-ELISA system still left about 10% false negative NPC (9). Further study of NPC patients' sera with negative result of the IgA/ [EBNA1+VCA-p18]-ELISA revealed that IgG to EA-p138, DNAse, and TK recognized in 30-40% of the samples, meanwhile EBNA1, EA(D), and ZEBRA in 70%, and to VCA-p40 and p18 were 100%. I these sera IgA antibodies recognized a more restricted pattern, confirming our prior studies (Fachiroh, Paramita et al., 2004).

We explored the diagnostic value of additional purified EA and VCA antigens based on their reactivity with NPC sera by IgG/EBV immunoblot. Result showed that recombinant and peptide-derived ZEBRA antigens showed better performance compared to DNAse and VCA-p160 recombinant proteins and peptides derived from VCA-p160. These results were in agreement with

IgG/ EBV immunoblot detection showing that ZEBRA was recognized (70%) in more samples compared to DNAse (40%). VCA-p160 usually is poorly resolved in the immunoblot analysis. Furtheron, recombinant ZEBRA showed lower sensitivity compared to OTP513 peptide derived from the ZEBRA N-terminus, even though the specificity are similarly low. This result is in contrast to Dardari et al. showing that recombinant full length ZEBRA showed better sensitivity, and comparable specificity to peptides representing DNA binding and activation sites of this protein (6, 7).

Major EA(D) complex antigens p47/54 (BMRF1) and p138 (BALF2) show 70% recognition in our IgG/EBV immunoblot detection of NPC with low IgA/[EBNA1+VCA-p18]-ELISA samples. This was recently explored by Paramita et al. (28), indicating that multiple EBV antigens required for developing IgA-ELISA for NPC diagnosis. For some proteins, recombinant full-length antigen shows better serological reactivity compared to synthetic peptides which represent only a short span of amino acid epitopes and lack most conformational epitopes, (6, 7, 13, 18).

van Grunsven et al. (34-36) have characterized VCA-p18 and VCA-p40 proteins encoded by BFRF3 and BdRF1, respectively. These two proteins are true late antigen and become part of virion structure. PEPSCAN analysis defined immunodominant epitopes of VCA-p18 within the C-terminal region (35). The VCA-p18 combi-peptide IV is used in our prior studies to develop EBV-ELISA systems (9). A monoclonal antibody (OT41A) raised against BdRF1 showed recognition on amino acid 275-300 of VCA-p40 (36) , but PEPSCAN analysis with human sera on the complete set of overlapping peptides of VCA-p40 failed to show significant and reproducible epitope reactivity in human sera (Middeldorp, unpublished data). The failure to identify immunodominant epitope of VCA-p40 in human sera indicates that the recognition as observed with full length VCA-p40 on immunoblot may relate to interaction with conformational rather than sequential epitopes in contrast to BFRF3.

Therefore, we have cloned a full length BdRF1 and a BFRF3-encoded immunodominant epitope into the pQE-Tri plasmid system (pQE-Tri-p40+18), aiming to improve epitope recognition in our ELISA-system for NPC diagnosis. The pQE-expression system is controlled by lac-operon, contains β-lactamase gene encoding for ampicilline resistance and 6x his-tag coding sequence either at 5' or 3' to the cloning region. Cloning gene sequence in the pQE-Tri system enable the intended recombinant protein to be expressed in E.coli, insect or mammalian cell (30). The expression of recombinant protein in E.coli provides many advantages related to cost-efficiency (21). However, compared to eukaryotic expression system, prokaryotes, do not have capacity for post-translational modification, such as phosphorylation and glycosylation. Bacteria tend to express recombinant protein as inclusion bodies, being aggregates, not folded in the native natural way. However, this aids their purification as inclusion bodies and refolding may be accomplished in later renaturation steps. Purification of recombinant protein expressed in E. coli should be done carefully; removing all cellular (read: contaminant) proteins, because humans commonly have antibody responses to bacterial antigens, causing a false positive read-out in serological assays. This condition may be avoided by using other expression systems such as insect cells (22).

We succesfully constructed and expressed in *E.coli* a fusion protein containing the full length BdRF1 and part of the BFRF3 gene sequence encoding immunodominant epitopes. The recombinant protein was detected at the expected MW of about 55KDa by both anti-VCAp18 and anti-VCAp40 specific antibodies and remained mainly in the insoluble (inclusion body) fraction. This allowed wash-away many soluble *E.coli* contaminants prior to the specific purification step. Unfortunately, the expression of recombinant protein in Dh5α strain was relatively low. We attempted to use different *E.coli* strains, SG13009 and M15, as both strains permitted high-level expression by having pREP4 repressor to ensure tight protein expression (30), but this did not

improve the fusion protein expression level (see fig.3). IPTG failed to enhance the expression of recombinant protein (see fig.3 and 4 for IPTG induction in the three *E. coli* strains tested).

Prior to purification, repeated freeze-thawing and sonication, was required to permeabilize and remove cell-membrane in the presence of lysozyme (fig. 5 and 7). High concentration of ureum was needed to ensure complete solubilization of the recombinant protein. Additional lysate-filtering and protease inhibitors were proven to reduce cell contamination and recombinant protein degradation (fig.7). The histidine-tag (positively charged) should become available by the urea-induced protein unfolding (denaturation), thereby permitting binding to nickel (negatively charged) on the column. The gradual increase of imidazole concentration (a positively charged histidine mimick) from loading, washing, and elution buffer, should release the histidine-tag binding from the nickel matrix. At the end, the protein of interest (VCAp40+18) was obtained in pure form (Figure 7, lanes 11-12), but unfortunately the purification efficiency was low. This is most probably caused by sub-optimal binding efficiency between his-tag and nickel column (fig. 7, lane 7,8), where the VCAp40+18 protein was detected in the washing buffer. We have tested several IMAC column matrices to replace the Nickel loaded agarose matrix but did not find better result (data not shown). Further work is still needed to achieve high protein expression, as well as improved purification. However, the purified VCAp40+18 protein could be used for initial exploration of its diagnostic value in acute IM and malignant NPC.

During acute infection (IM), IgM responses are directed to EBV early (EA) and late (VCA) phase antigens. These IgM responses subsequently disappear during convalescence, therefore being suitable for diagnosis of acute IM (1, 27). Commercial strip tests and ELISAs for IM diagnosis have become available for recent years. Recently Paramita et al. (29) and Buisson et al. (2) evaluated the use of different brands of commercial strips, RecombLine (Mikrogen

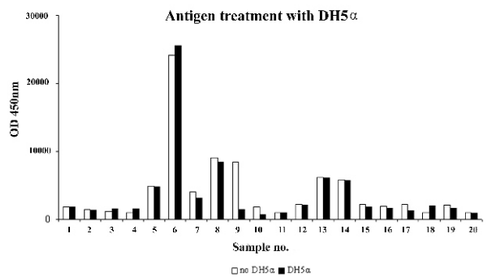

Fig. 6. Addition of pQE-Tri plasmid free-DH5α cell lysate to VCAp40+18 recombinant antigen prior to coating compared to those of without cell lysate. Data showed that no significant difference between OD450nm of IgA/VCAp40+18 and IgA/VCAp40+lysate, which indicated that no IgA antibody generated to Dh5α cellular protein, and extra other band retain in the elution fraction will not interfere with the ELISA assay.

GmbH,Neuried, Germany) and EBV IgM/IgG Blot 3.0 assay (Genelabs Diagnostics, Singapore), respectively. Each strip contains four different EBV antigens, with extra fifth band of VCA-p18 antigen on RecombLine. Both assays are in agreement by showing IgM reactivity to EA(D) p47/54, EA-p138, and lesser to VCA-p23, whereas in the RecombLine an addition weak reactivity was found in IgM to VCA-p18 (29), confirming prior data in IgM/VCA-p18 ELISA (35). Our study shows that IgM response to VCAp40+18 strip was detectable in all ten samples (100%) tested, compared to IgM/ VCA-p18 synthetic peptide with detection of 66.67% (6 of 9) and 7 of 9 (77.78%) for in-house

indirect and commercial capture ELISAs, respectively (table 2). This result indicates that VCAp40 has diagnostic value for IM diagnosis at the acute phase.

The purified VCAp40+p18 protein was further used for NPC diagnosis in an IgAELISA format, in a panel tested extensively for anti-EBV IgA and IgG reactivity by immunoblot and ELISA (9, 11). NPC samples with negative result in IgA/ [EBNA1+VCA-p18]-ELISA showed overall 100% IgG reactivity to VCA-p40 and VCA-p18 in EBV-blot but lower to other EBV antigens (table 1). IgA/ VCAp40+18 ELISA detected 63.6% and 95% of low and high IgA/[EBNA1+VCA-p18]-ELISA samples, respectively. Serum samples from healthy donors (n=12) were all negative in the IgA/ VCAp40+18 system. Overall, the new ELISA system was able to detect 33 of 42 NPC samples tested (78.6%). When we add the IgA/ VCAp40+18 to IgA/ [EBNA1+VCAp18]-ELISA in our NPC serology systems, the sensitivity for diagnosing NPC may rise from 90% to 96.3% without comprosing the specificity.

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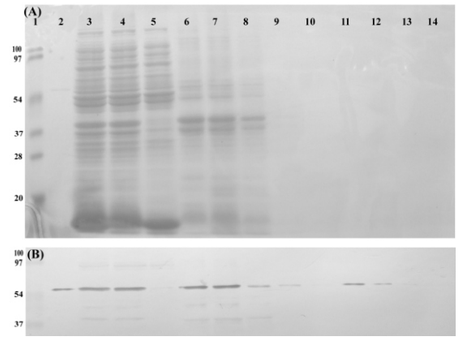

Fig. 7. Flow-through of VCAp40+18 protein purification with additional steps to pre-loading material filtering in 0.22 µm subsequently added with protease inhibitors run in 10% SDS-gel (A) Immunoblot detection by IgG antibody from healthy carrier reference (JM; 1:100) (B). Result shows less cellular contaminants in elution fraction (compared to fig. 4A), but still with low efficiency of protein binding to column matrix (see lane 7 and 8). (1. molecular weight marker- weight marker indication are written at the left side of the figure (in kDa); 2. purified VCAp40+18 from previous experiment, 3. freeze-thaw; 4. pellet after 1st sonication; 5. supernatant after 1st sonications; 6. pellet after 2nd sonication; 7. pre-load material after additional step; 8. flow trough loading; 9,10. flow trough of 2 times washings; 11,12,13: eluate fractions; 14 column material afterwards)

Table 2. IgM detection on acute infectious monucleosis samples (n=10)

sample No.	IgM/ VCA-p40-18 blot	IgM/ VCA-p18 ELISA	IgM/VCA-ELISA
1	+	+	+
2	+	+	+
3	+	-	+
4	+	-	+
5	+	+	+
6	+	-	-
7	+	+	-
8	+	+	+
9	+	NT	NT
13	weak	++	+

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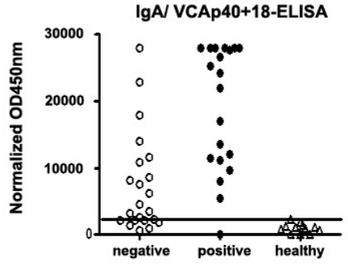

Fig. 8. IgA/VCAp40+18- ELISA on NPC samples negative (n=22) and positive (n=20) in IgA/[EBNA1+VCA-p18]-ELISA, compared to normal healthy donor plasma samples (n=12). Horizontal line showed Cut-off value (CoV : 2,351) of IgA/VCAp40+18-ELISA. The IgA/VCAp40+18 is able to detect 14/22 (63.6%) and 19/20 (95%) of samples with negative and positive IgA/[EBNA1+VCAp18] respectively.

In summary, our results show the value of recombinant VCAp40+18 for diagnosis of acute IM and NPC. For acute IM, the new recombinant protein may be applied in immunoblot strip or IgM-ELISA format. For NPC diagnosis, we propose to use EBNA1 synthetic peptide added to VCAp40+18 recombinant protein as mix or separate assay for EBV-IgA detection.

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CHAPTER 5

Dried-Blood Sampling for Epstein-Barr Virus Immunoglobulin G (IgG) and IgA Serology in Nasopharyngeal Carcinoma Screening

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ABSTRACT

Dried-blood (DB) samples on filter paper are considered clinical specimens for diagnostic use because of the ease of collection, storage, and transport. We recently developed a synthetic-peptide-based immunoglobulin A (IgA) (EBNA1 plus viral capsid antigen [VCA]-p18) enzyme-linked immunosorbent assay (ELISA) for nasopharyngeal carcinoma (NPC) screening. Here, we evaluate the use of two filter papers for DB sampling, i.e.,Schleicher & Schuell (S&S) no. 903 and Whatman no. 3; the DB samples were either taken directly from a finger prick or spotted from a Vacutainer blood collector. The elution of DB samples on filter paper was optimized and tested for IgG and IgA reactivity by ELISA (EBNA1 plus VCA-p18) and compared to simultaneously collected plasma samples. The results showed that both types of filter paper can be used for sample collection in NPC diagnosis by using either finger prick or blood spot sampling. Both DB sampling methods produced comparable ELISA (EBNA1 plus VCA-p18) results for IgG and IgA reactivity in 1:100-diluted plasma samples. DB samples of whole blood or finger prick blood show correlation coefficients (r^2) of 0.825 to 0.954 for IgA on S&S no. 903 filter paper, 0.9133 to 0.946 for IgA on Whatman no. 3 filter paper, 0.807 to 0.886 for IgG on S&S no. 903 filter paper, and 0.819 to 0.934 for IgG on Whatman no. 3 filter paper. Using plasma IgA as a reference, DB sampling showed sensitivities and specificities of 75.0 to 96.0% and 93.5 to 100%, respectively. DB samples could be stored at 37^{0}C for 1 to 4 weeks on S&S no. 903 filter paper and 1 to 6 weeks on Whatman no. 3 filter paper without a significant loss of reactivity, with provision of transport options for tropical conditions. IgA proved to be more stable than IgG. Whatman no. 3 filter paper is a more economical yet diagnostically comparable alternative to S&S no. 903 filter paper. Finger prick DB sampling is proposed for NPC diagnosis, particularly for remote hospitals and field screening studies.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a disease with remarkable geographic and racial distribution worldwide. NPC is a rare disease in many parts of the world, including in Europe and North America, with an incidence below 1 per 100,000 persons. High-incidence regions are located mainly in southern China (25 per 100,000 persons per year in the Guangzhou area), Taiwan, and some Southeast Asian countries (48). There are also areas of intermediate incidence (3 to 8 per 100,000 persons per year), e.g., North Africa, Alaska, Greenland, and highly populated Asian countries, such as Vietnam and Indonesia (5, 48). In Indonesia, especially in central Java, undifferentiated NPC (WHO type III) ranks among the most common types of cancer. In the Yogyakarta province, hospital-based data showed NPC to be ranked the number 1 cancer in males and the number 3 cancer in females (41), with regional villages representing hot spots with high NPC incidence (unpublished data).

NPC WHO type III is virtually 100% associated with the Epstein-Barr virus (EBV). EBV infection in NPC tumor cells displays a type II latency pattern by the expression of EBV EBNA1, LMP1, LMP2, and noncoding EBV-encoded RNA and BamHI A rightward transcript RNA (4, 34), with the additional expression of the BARF1 oncogene (4, 42). EBV was first linked with NPC on the basis of the serological observations made by Old and colleagues in 1966 (36) and further elaborated by Henle and Henle (20). NPC is characterized by aberrant immunoglobulin G (IgG) and particularly IgA responses directed against various latent and lytic EBV antigens (13). These aberrant responses have diagnostic relevance in screening for early-stage and posttreatment monitoring (7, 22, 23, 43). The diagnosis and screening of NPC are done mostly by indirect antibody detection using cell spot slide tests, one of the earliest serology methods developed, which to date is still used as a “gold standard” (8, 35). However, these slide-based assays are subjective and cumbersome, making their application in mass screening inconvenient (11, 23).

Enzyme-linked immunosorbent assay (ELISA) techniques provide a promising alternative with potential for automation and mass screening (11, 14). Recently, we developed a well-standardized IgA EBV ELISA for the primary diagnosis of NPC using a combination of multiepitope EBNA1- and viral capsid antigen (VCA)-p18-derived synthetic peptides in a single-well format and combined it with the detection of IgG reactivity to the EBV immunoblot strips for the confirmatory test (herein referred to as the IgG and IgA EBV ELISA). For field studies, a simple sample collection and transport system is desirable. The use of filter paper for blood collection and analysis was implemented as early as the 1960s by Guthrie et al. using dried-blood (DB) samples for newborn phenylketonuria.

MATERIALS AND METHODS

Blood, plasma, and DB samples. Blood from healthy donors (n=98) was taken from volunteers in the Yogyakarta region of Indonesia. NPC samples (n=42) were taken from first-visit patients enrolled in the ear, nose, and throat clinic at Sardjito Hospital in Yogyakarta as part of a standard serology screening procedure (14). NPC status was confirmed for all samples by computer tomography scanning and pathological biopsy examination. In addition, the EBV-positive status of the tumors was confirmed by immunohistochemistry staining using OT1X antibody directed to EBNA1 (7). For all healthy blood donors, parallel samples were taken from both a fingertip and vein in the arm, while for NPC patients, samples were taken from only the arm.

Sample collection. FP samples were taken by pricking the middle-finger tip with a lancet (Baxter, United Kingdom) after it was cleaned with 70% ethanol. The blood was allowed to drip directly onto S&S no. 903 (Schleicher & Schuell, Germany) and Whatman no. 3 (Whatman, United Kingdom) filter papers until a circle with a diameter of about 10 mm formed. BS samples were prepared by drawing 100 ml whole blood from a heparinized Vacutainer vial and by spotting it onto S&S no. 903 and Whatman no. 3 papers. Plasma samples were prepared from the same Vacutainer by whole-blood centrifugation at 1,800 rpm for 15 min and subsequently by plasma isolation. The FP, BS, and plasma samples were stored at -20^{0}C until use. The BS samples were also stored at elevated temperatures where indicated below.

Plasma elution from DB samples. Using a paper puncher, 25-mm^2 BS disks were cut. One disk was immersed in sample buffer (1% bovine serum albumin, 0.1% Triton X-100, and 0.05% Tween 20 in phosphate-buffered saline). The elution of IgA was optimized by variation (i) of the volume of the sample buffer, (ii) in the elution solvent, and (iii) in the incubation temperature and time, independently for Whatman no. 3 and S&S no. 903 papers, to achieve an optical density value at 450 nm (OD_{450}) comparable with that of the 1:100-diluted plasma samples in our standard EBV ELISA (14).

EBV serology tests. The standard serology test consisted of our IgG and IgA EBV ELISA for NPC diagnosis/screening (13, 14). The EBNA1 and VCA-p18 synthetic peptides were made based on the predicted immunodominant epitope defined by Pepscan analysis (30) and prepared as described elsewhere (28, 30, 47). IgG and IgA EBV ELISAs were performed as described previously, and they used EBV-seropositive and seronegative sera as controls in each run (14). All samples were tested in duplicate. The cutoff value (CoV) was determined to be 0.3536, according to receiver operating characteristic curve analysis, defined as the threshold value optimally separating “healthy” samples from “disease” samples (31). The OD_{450} value of each sample was corrected with that of a negative plasma background reaction as described in detail before (10, 14).

For the confirmation test, EBV immunoblot strips containing nuclear antigens from HH514.c16 cells chemically induced to produce the late lytic phase of EBV proteins were used to

detect IgG reactivity to the spectrum of EBV EBNA1 and lytic antigens. The strips were prepared and analyzed exactly as described previously (13, 29). Characteristic EBV antigens on blot strips were defined by known human reference sera and monoclonal/monospecific polyclonal antibodies (13). A sample was determined to have a "normal pattern" when IgG reactivity was detected against any combination of EBNA1 (BKRF1 [72 kDa]), VCA-p40 (BdRF1 [40 kDa]), ZEBRA (BZLF1 [36 plus 38 kDa]; fine doublet), and VCA-p18 (BFRF3 [18 kDa]). A sample was determined to have an "abnormal pattern" when IgG reactivity to an EBV antigen(s) other than those involved in the "normal pattern" was present.

DB sample stability. To evaluate the stability of stored BS samples on filter paper, we obtained several DB samples from four healthy individuals. Separately, 100 ml of blood from a heparinized Vacutainer was spotted onto either S&S no. 903 or Whatman no. 3 filter paper, dried overnight at room temperature (RT; 18 to 22^{0}C), placed in a paper envelope, and stored at -20^{0}C, 4^{0}C, RT, and 37^{0}C. In addition, RT and 37^{0}C incubations were measured to have similar relative humidities (±30%). Stored BS samples were processed with the IgG and IgA EBV ELISA using the optimized elution method for each type of paper. Evaluations were done once a week for 4 weeks and then at 2-week intervals for a period of 24 weeks.

Analysis. The descriptive statistical analysis values (means, medians, standard deviations) and correlation coefficients (r^2) were determined by comparing the individual IgG or IgA EBV ELISA results for the DB or plasma samples. The sensitivity and specificity of the DB samples were determined by using plasma IgA EBV ELISA results as the reference for the positive and negative values. After correction with the results of a negative plasma background reaction, OD_{450} values above 0.3536 were stated as "IgA positive" and values below 0.3536 as "IgA negative." Statistical analysis was done by GraphPad Prism version 4.03.

RESULTS

Plasma IgA and IgG EBV ELISA results. The samples from healthy donors were collected specifically for this study, while the NPC samples were obtained from patients with histologically confirmed NPC who were enrolled in the NPC treatment program at Sardjito Hospital, Yogyakarta, Indonesia (14). The NPC subjects (n= 42) were 71.1% male and 29.9% female, presenting disease stages III (41.7%) and IV (58.3%), with ages ranging from 18 to 70 years (<30 years, 20.6%; 31 to 40 years, 17.6%; 41 to 50 years, 26.5%; and > 50 years, 35.3%). Figure 1 shows IgG and IgA EBV ELISA results of all plasma samples used in this study. The means and standard deviations of the OD450 values were 1.879 and 0.643, respectively, for the IgG of the healthy donors and 3.135 and 0.359, respectively, for the IgG of the NPC patients, while they were 0.141 and 0.141, respectively, for the IgA of the healthy donors and 1.498 and 0.887, respectively, for the IgA of the NPC patients. The IgA EBV ELISA, using a CoV of 0.3536 (14), showed 94.9% (93/98) of healthy blood donors to be negative and 97.7% (41/42) of NPC patients to be positive, leading to 5.1% (5/98) of results potentially being false positive and 2.3% (1/42) of results potentially being false negative (Fig. 1). The IgG immunoblot confirmation test (13) showed that one of five false-positive healthy blood donor samples and one false-negative NPC patient sample had an "abnormal pattern" and four of five false positive healthy blood donor samples had a "normal pattern" compatible with the absence of NPC (data not shown). The results from the IgG and IgA EBV ELISA were almost completely in agreement with the clinicopathological diagnosis, showing 1 healthy donor out of 98 (1.02%) as being a candidate potentially at risk for NPC (no follow-up available).

IgA elution from DB samples. The optimization of IgA elution from DB samples was

done in several steps. First, we defined the optimal elution buffer composition, which is described in Materials and Methods. We then defined the optimal elution volume (200, 300, 400, 500, and 600 µl/25-mm^2 paper disk) and the incubation time in combination with the incubation temperature (4^{0}C and RT) for both S&S no. 903 and Whatman no. 3 filter papers. The optimal condition was defined as one in which OD450 values for IgA eluted from BS samples and for IgA eluted from 1:100-diluted plasma samples are comparable in a standard IgA EBV ELISA. The optimum sample buffer volume was 500 µl for S&S no. 903 filter paper and 400 µl for Whatman no. 3 filter paper. Figure 2B shows

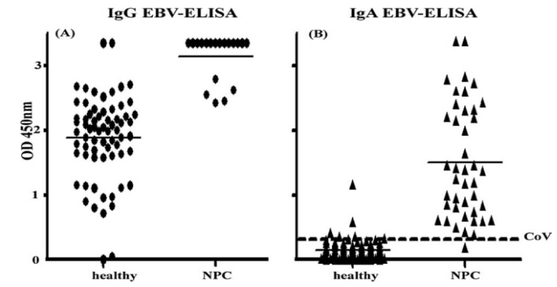

Fig. 1. IgG (A) and IgA (B) EBV ELISA results for healthy donorplasma samples (IgG, n=68; IgA, n=98) and NPC plasma samples (IgG, n=19; IgA, n= 42) used within this study. Medians are indicated by the straight lines.

the yield of IgA measured after elution from filter paper for different time periods, ranging from 0.5 to 24 h. IgA was optimally eluted after 1 h of incubation at RT for S&S no. 903 filter paper, with similar results obtained with additional samples (n=9). For Whatman no. 3 paper, IgA was optimally eluted after 4 h of incubation in 4^{0}C (Fig. 2B). The eluted IgA was stable for a period of 24 h at 4^{0}C and at RT (Fig. 2A and B). Similar results were obtained for IgG elution (data not shown).

Comparison of plasma, BS, and FP IgG and IgA EBV ELISA results. After the optimization of IgA elution, the panel of DB and plasma samples was tested by the IgA EBV ELISA according to the standard protocol (14). The DB samples used in this study had already been stored sealed for up to 12 months at -20^{0}C prior to use. The OD_{450} values for the DB (BS and FP) samples were compared to those for the 1:100- diluted plasma samples. In parallel, we analyzed eluted-IgG EBV ELISA results from both BS and FP samples on both S&S no. 903 and Whatman no. 3 filter papers. To compare the IgG and IgA EBV ELISA results of both DB and plasma samples, we analyzed the correlation coefficients (r^2) of the OD_{450} values for IgA and IgG for the BS versus plasma, FP versus plasma, and BS versus FP samples for both filter papers. In general, DB samples from S&S no. 903 and Whatman no. 3 filter papers produced OD_{450} values in IgA and IgG EBV ELISA highly comparable to those of the 1:100-diluted plasma samples. Individual r^2 values of the tests are presented in Table 1.

DB sampling sensitivity and specificity. DB sampling sensitivity and specificity for BS and FP samples on both types of filter paper were defined by the IgA EBV ELISA only, the preferred method for NPC screening (14). Plasma IgA levels from both NPC and healthy panels were used as references for determining positive or negative status, as well as to define false-positive and false-negative results for each filter paper analyzed. Sensitivity and specificity values for each type of filter paper are shown in Table 2. For S&S no. 903 filter paper, the sensitivity and specificity of the BS

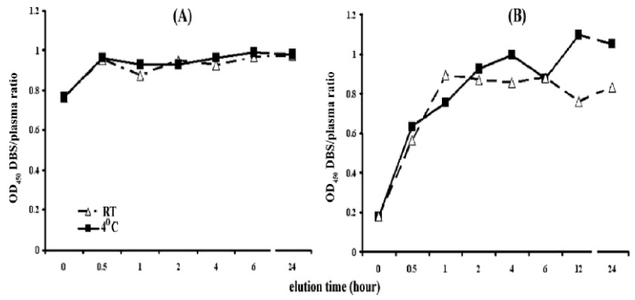

Fig. 2. Dynamic of IgA elution during a 24-h incubation of BS samples on S&S no. 903 (A) and Whatman no. 3 (B) filter papers. The optimal IgA elution for S&S no. 903 filter paper (500 µl sample buffer/25-mm2 filter disk) was 1 h of incubation at RT, while for Whatman no. 3 filter paper (400 µl sample buffer/25-mm^2 filter disk), it was 4 h of incubation at 4^{0}C. Overall, IgA was stable for 24 h with RT and 4^{0}C incubation.

samples were 96.0 and 93.6%, and those of the FP samples were 80.0 and 100%, respectively. For Whatman no. 3 filter paper, these values were 89.2 and 97.3 for the BS samples and 75.0 and 97.1% for the FP samples, respectively.

DB sampling stability. Parallel BS samples (n= 4) were stored in envelopes at -20^{0}C, 4^{0}C, RT, and 37^{0}C for 24 weeks. At different time points, one sample was eluted and tested by the IgA and IgG EBV ELISA and compared to a 1:100-diluted plasma sample. A normalized value was

Table 1. Correlation coefficients between OD450 values of plasma and DB samples tested by our IgG and IgA EBV ELISA

ELISA (total no. Of healthy donors and NPC patients plasma samples)	Filter paper	r^2 between OD_{450} of indicated samples (no of samples tested)		
		Plasma vs BS	Plasma vs FP	BS vs FP
IgA (150)	S&S no. 903	0.954 (82)	0.836 (63)	0.825 (63)
	Whatman no. 3	0.913 (78)	0.913 (78)	0.929 (78)
IgG (87)	S&S no. 903	0.886 (55)	0.807 (35)	0.886 (35)
	Whatman no. 3	0.819 (37)	0.865 (31)	0.934 (31)

obtained by defining the ratio of OD_{450}s for BS and 1:100-diluted plasma samples in the same test. Figure 3 shows one of the four samples tested by our IgG and IgA EBV ELISA on S&S no. 903 and Whatman no. 3 filter paper. When stored at -20^{0}C and 4^{0}C, both types of paper showed relatively stable IgA and IgG values until the 24th week. IgA and IgG reactivities of the samples stored on either type of filter paper at RT showed less stability than those of samples stored at 37^{0}C, with decreasing stability observed at week 2 for IgA and at week 0 to 1 for IgG. IgA was more stable than IgG, with decreasing reactivity from 4 (S&S no. 903 paper) and 6 (Whatman no. 3 paper) weeks onwards.

DISCUSSION

EBV serology is commonly used to facilitate the diagnosis of suspected NPC and is

proposed for large field screening and epidemiology survey studies (35, 49). NPC risks are associated with the elevated responses of IgG and particularly of IgA antibodies to certain EBV antigens. Most people in Southeast Asia are first infected by EBV in early childhood, reflected by a nearly 100% seropositivity for IgG to EBV VCA and EBNA1. Figure 1 shows IgG EBV reactivity in healthy blood donors overlapping with IgG levels in NPC patients, thus precluding diagnostic use. On the other hand, IgA reactivities differed more significantly between healthy donors and NPC patients, in agreement with prior studies (14, 20, 23). The presence of IgA to EBV VCA suggested the reactivation of EBV in epithelia, paralleling NPC development. A 15-year follow-up study in China recently revealed that elevated IgA responses to EBV VCA become apparent within a 2-year "window" period before clinical manifestation (22). Another study showed elevated IgA responses to EBV VCA at 16 to 41 months prior to the clinical manifestation of NPC (50). In those previous studies, an EBV slide test was used, which is suboptimal for screening purposes. ELISA is considered a more suitable tool for serological screening because of its relatively low cost, standardization of reagents, and suitability for automation, allowing the processing of large numbers of samples under identical conditions (11, 14). For the serodiagnosis of NPC in a high-risk population in Indonesia, we recently developed a one-step IgA EBV ELISA by combining immunodominant epitope peptides from EBNA1 and VCA-p18 (14). In agreement with others, we demonstrated that IgA to EBNA1 and VCA-p18 is a highly reliable marker for NPC screening (8, 40). The use of synthetic peptides greatly improves the standardization of EBV ELISA. Our IgA EBV ELISA showed that in a panel of freshly collected blood samples, 5 of 98 (5.1%) healthy blood donors were above the CoV and 1 of 42 (2.4%) NPC patients was below the CoV. These aberrant samples were subsequently tested by using immunoblotting as the confirmation test assessing the EBV IgG diversity according to Fachiroh et al. (13). Confirmation testing revealed that one of five healthy blood donors with elevated IgA values had an "abnormal pattern" but that the other four samples had a "normal pattern." The one NPC patient with a low IgA EBV value presented an "abnormal pattern," suggestive of NPC. This sample was later confirmed by positive EBVencoded RNA staining of tumor tissue. In brief, our screening methodology combining IgA EBV ELISA and a confirmation test, IgG EBV immunoblotting, confirmed the diagnoses of 97 of 98 (98.98%) healthy donors and all (100%) of the NPC samples tested within this study.

In some parts of Java Island in Indonesia, as well as in other parts of the vast Indonesian archipelago, there are "hot spots" of NPC, most of them in rural areas with geographic barriers, and they are localized at a distance from the central diagnostic laboratory. In current practice, most NPC patients first present to the clinic with late-stage (III/IV) disease involving a large primary tumor mass and lymph node metastasis, as also seen in our NPC panel (see Results). When diagnosed and treated early, NPC can be effectively treated with radiotherapy, leading to highly improved cure rates. Therefore, it is desirable to have a simple sample collection system in place for screening and diagnosis in remote populations at risk of NPC. It is considered relevant to combine a reliable screening assay with a simple sampling method. The use of filter paper sampling is proposed to be combined with FP bleeding to replace blood drawing from the arm. This combines simple sampling with the ease of transportation for subsequent testing in a reference laboratory. S&S no. 903 filter paper (known as "Guthrie paper") is a standard sampling paper and is widely used for many types of analyses, including those for DNA (9), RNA (1), protein (21, 25, 37), and chemical substances (19, 38). Whatman no. 3 filter paper is a thick membrane with tight pores used as a fine particle filtration device (Whatman product information), while S&S no. 903 filter paper is a special liquid specimen collector made of cotton fiber which has the capacity to absorb/release liquids efficiently. Whatman no. 3 filter paper required longer time than S&S no. 903 filter paper to absorb/ elute blood (Fig. 2).

When Whatman no. 3 paper is used, attention is required during the collection of FP samples to ensure that spots are completely saturated with blood. Whatman no. 3 filter paper is a good alternative to S&S no. 903 filter paper, with its low cost, local availability, and comparable sensitivity.

The elution of Ig from the DB samples was highly reproducible by using the same buffer used to dilute plasma in our standard ELISA protocol. The eluted Ig solution from the DB samples could be stored at -20°C for a few days without reducing its reactivity (data not shown). Thus, it is possible to prepare samples several days in advance of testing. All Ig samples eluted from the DB samples either FP or BS samples could be detected by ELISA. Table 1 shows an excellent correlation between OD450 values from the two steps of the IgG and IgA EBV ELISA for either the BS or FP samples on both paper types. By using plasma IgA as the reference, the sensitivity and specificity for the BS and FP samples from both types of filter paper were between 75.0 and 100%, respectively, as shown in Table 2. False-positive and/or -negative samples were found among those with values close to the cutoff point, indicating the necessity for precise elution volume and time. Table 2 also shows that the FP samples have lower sensitivities than the BS samples, and yet they have comparable specificities. This may reflect the occasionally limited volume of blood collected on the filter paper spot, indicating that FP sampling needs to be performed with care.

The antibody contained in the DB samples may decay in a humid atmosphere (17), but

Table 2. Sensitivity and specificity of DB samples compared to those of plasma samples in an IgA EBV ELISA[a]

Filter paper	DB sample type [a]	No. of samples with indicated ELISA results				Sensitivity (%)[d]	Specificity (%)[e]
		IgA positive[b]	IgA negative[c]	False positive	False negative		
S&S no. 903	BS	24	58	4	1	96.0	93.5
	FP	4	59	0	1	80.0	100.0
Whatman no. 3	BS	33	72	2	4	89.2	97.3
	FP	12	66	2	4	75.0	97.1

[a] For S&S no. 903 filter paper, 82 BS and 63 FP samples were tested. For Whatman no. 3 filter paper, 105 BS and 78 FP samples were tested.
[b] IgA-positive samples were defined as samples with OD_{450} values above 0.3536 (13).
[c] IgA-negative samples were defined as samples with OD_{450} values below 0.3536.
[d] Sensitivity was defined as the number of IgA-positive samples divided by the sum of the number of IgA-positive and false-negative samples.
[e] Specificity was defined as the number of IgA-negative samples divided by the sum of the number of IgA-negative and false-positive samples.

when stored properly, DB sampling will ensure Ig stability for a long period. We studied the stability of IgG and IgA in BS samples stored in paper envelopes, without desiccant, under different temperatures for 24 weeks. Results showed that IgG and IgA were relatively stable when stored at 4°C and -20°C until the 24th week, in agreement with other serological studies for EBV (25), HIV (18), and measles virus (39). This demonstrates that DB samples will retain their biological contents when stored at a low temperature.

When stored at RT and 37°C, Whatman no. 3 filter paper provided somewhat better IgG and IgA stability than S&S no. 903 filter paper. The DB samples stored at 37°C maintained IgA for 4 to 6 weeks and IgG for 1 week (Fig. 3). This allows sufficient time for the transport of a sample in a tropical atmosphere from a regional hospital or rural area to the central laboratory by standard mail. Storage at RT provided lower IgA and IgG stability than storage at 37°C. This is dissimilar with other findings (3, 25, 45), which indicated that DB samples stored at RT had better antibody (IgG and IgE)

stability than those stored at higher temperatures. McDade et al. (25) showed that DB samples were stable in IgG VCA-p18 ELISAs for at least 8 weeks at 4°C and RT but deteriorated after 1 week at 37°C. Our data showed similar results for IgG DB samples stored in 37°C but longer IgA stability for DB samples collected on both paper types. The specific mucosal origin of IgA may provide enhanced stability under "high-stress" conditions like bacterial contamination or proteolytic degradation.

To obtain longer antibody stability on DB specimens, it was suggested that the humidity needs to be controlled (3). Mei et al. (26) emphasize the importance in avoiding humidity by storing DB samples in ziplock bags with desiccant since moisture may harm the specimens by inducing bacterial growth or altering the elution time of the specimens. Dried BS specimens stored in ziplock bags with desiccant can be stored at -20°C for many weeks or years (3, 6, 46).

In summary, our data indicates that DB samples obtained from FP sampling or from a Vacutainer tube may be transported at ambient temperatures by regular mail without the loss of sensitivity and specificity. IgA DB samples are stable for months when stored in cold temperatures. Both S&S no. 903 and Whatman no. 3 filter papers can be used to replace fresh plasma sample type in the NPC field screening program, with Whatman no. 3 providing a more economical alternative.

DB samples for IgA EBV serology can be prepared by applying a few drops of fresh blood drawn by venipuncture or by FP sampling using a lancet. FP sampling allows access to individuals for whom drawing blood by venipuncture may be problematic, such as children and elderly people. FP DB sampling may be a more practical sampling method, as it is inexpensive and does not require trained personnel. DB samples can be sent at ambient temperatures to a research laboratory or a central hospital for testing. For long periods of storage, DB samples require less space than plasma samples. With these advantages, FP DB samples may replace fresh blood as samples for NPC serological studies. Therefore, FP DB sampling is proposed as a tool for EBV/

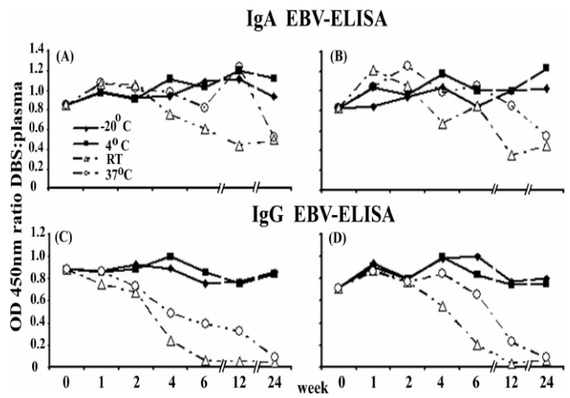

Fig. 3. IgA EBV ELISA results showing the stability of IgA in DB samples on S&S no. 903 (A) and Whatman no. 3 (B) filter papers, and IgG EBV ELISA results showing the stability of IgG on S&S no. 903 (C) and Whatman no. 3 (D) filter papers over a period of 24 weeks in storage at -20°C, 4°C, RT, and 37°C. Results showed that IgG and IgA in both filter papers remained stable until the 24th week when DB samples were stored at -20°C and 4°C for 4 to 6 weeks for IgA and at 37°C for 1 week for IgG and for less time when the samples were stored at RT.

NPC screening in combination with IgA EBV ELISA, providing a standardized and economical method. The DB sampling method enables population-based screening in remote areas, which is important in finding early-onset NPC cases.

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CHAPTER 6

Diagnostic Value of Measuring Epstein-Barr Virus (EBV) DNA Load and Carcinoma-Specific Viral mRNA in Relation to Anti-EBV Immunoglobulin A (IgA) and IgG Antibody Levels in Blood of Nasopharyngeal Carcinoma Patients from Indonesia

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in Southeast Asia and is strongly associated with Epstein-Barr virus (EBV). We investigated the primary diagnostic value of circulating EBV DNA and anti-EBV immunoglobulin G (IgG) and IgA levels in Indonesian NPC patients (n =149). By a 213-bp Epstein-Barr virus nuclear antigen 1 (EBNA1)-based real-time LightCycler PCR, 72.5% of patients were positive for EBV DNA in whole blood, with 29.5% having levels above a previously determined clinical cutoff value (COV) of 2,000 EBV DNA copies/ml, the upper level in healthy carriers. In a 99-bp LightCycler PCR, 85.9% of patients were positive and 60.4% had levels above the COV. This assay quantified a significantly higher EBV load than the 213-bp PCR assay (P <0.0001), suggesting that circulating EBV DNA is fragmented. Using data from 11 different studies, we showed a significant inverse correlation between PCR amplicon size and the percentage of patients positive for circulating EBV DNA (Spearman's rho P < 0.0001). EBV DNA loads were unrelated to anti-EBV IgG or IgA levels, as measured by VCA-p18 and EBNA1-specific synthetic peptide-based enzyme-linked immunosorbent assays. The presence of circulating tumor cells was assessed by amplification of BamHI-A rightward frame 1 (BARF1) mRNA, a viral oncogene abundantly expressed in EBV-carrying carcinomas but virtually absent from EBV-associated lymphomas. Despite high EBV DNA loads and the presence of EBNA1 and human U1A small nuclear ribonucleoprotein mRNA, BARF1 mRNA was never detected in blood. We conclude that amplicon size significantly influences EBV DNA load measurement in NPC patients. The circulating EBV DNA load is independent of serological parameters and does not reflect intact tumor cells. The primary diagnostic value of the EBV DNA load for the detection of NPC is limited.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world; but it occurs endemically in southern China, Hong Kong, Singapore, and some other parts of Southeast Asia with high incidences (>17 cases/100,000 populations/year) (17). NPC has a strong etiological link with Epstein-Barr virus (EBV), a ubiquitous herpesvirus that establishes a lifelong, mainly asymptomatic infection in 90% of the world's population (27, 58). EBV is clonally present in virtually 100% of undifferentiated NPC cases, and the virus may express a restricted number of latent genes. These include the small EBV-encoded nonpolyadenylated RNAs (EBER1 and EBER2), the Epstein-Barr virus nuclear antigen 1 (EBNA1), two latent membrane proteins (LMP1 and LMP2), the noncoding BamHI-A rightward transcripts (BARTs), and BamHI-A rightward frame 1 (BARF1) (27, 37, 40, 42). EBNA1 is essential for maintenance of the viral genome and its anchoring to host chromosomes. The LMP1, LMP2, and BARF1 proteins all have transforming properties in epithelial cells in vitro (26, 45, 46, 64, 65). Whereas EBNA1, LMP1, and LMP2 are expressed in both EBV-linked lymphomas and carcinomas, BARF1 is a viral oncogene that is almost exclusively transcribed in EBV-positive carcinomas and that is virtually absent from EBV-associated lymphomas (5, 12, 21, 69). Carcinoma-associated EBV activity in NPC patients may be reflected in the circulation by a typical anti-EBV serological profile and increased viral DNA levels. In comparison to healthy EBV carriers, NPC patients generally show strong immunoglobulin G (IgG) and especially IgA reactivities to EBV early antigens, viral capsid antigens, and EBNA1 (22). Serodiagnostic assays based on defined EBV-derived epitopes may facilitate population-based screening aimed at the early identification of NPC patients. Monitoring of EBV DNA and RNA parameters in blood, if it is proven to be sensitive and specific, could be potentially useful for confirmation of initial serodiagnostic risk stratification in mass surveys.

Studies on monitoring of the EBV DNA load in the circulation of NPC patients mostly originate from Hong Kong (7, 30, 31, 34, 59); but elevated EBV DNA loads can also be found in the plasma of Thai (38, 48), Taiwanese (25), Chinese (47), and some Italian (41) NPC patients. The reported percentage of NPC patients positive for EBV DNA in peripheral blood, however, varies strongly from approximately 30 to 98%; and many studies report sensitivities below 90% and very low EBV DNA loads in a significant proportion of patients (9, 24, 25, 38, 48).

Therefore, the aim of this study was to determine the primary diagnostic value of circulating EBV DNA loads in a large cohort of Indonesian NPC patients (n=149) from the Yogyakarta region, where NPC represents the number one malignancy in males and the number four tumor in females (50). We tried to find an explanation for the large variations in peripheral blood EBV DNA positivity in NPC patients reported thus far by using different DNA target sizes in PCR. We used unfractionated whole blood, a clinical specimen type previously shown to be diagnostically relevant in transplant recipients, human immunodeficiency virus-positive and AIDS patients, and Burkitt's lymphoma patients (51, 57). In these populations a cutoff value of 2,000 EBV DNA copies/ml blood clearly demarcates patients with EBV-positive tumors from healthy EBV-seropositive carriers, in whom the loads were invariably below this value (54, 55). The use of whole blood might increase the sensitivity of detection by combining plasma and cellular fractions and, furthermore, enables the parallel detection of EBV mRNA. At present it is not clear whether NPC tumor cells can enter the circulation. In order to detect possible circulating NPC tumor cells, whole-blood specimens were studied for the presence of EBNA1 and BARF1 mRNA. In contrast to EBV DNA, which may be present at low levels in the circulation of healthy EBV-seropositive carriers, BARF1 RNA may directly reflect NPC-related EBV transcriptional activity, whereas EBNA1 mRNA may be found in both circulating latently EBV-infected B cells undergoing cell division (23) and intact NPC tumor cells. EBV mRNA detection in peripheral blood has hitherto not been studied as an NPC marker, but we recently showed its diagnostic potential in nasopharyngeal tissue biopsy specimens (5, 21) and nasopharyngeal brushings (S. J. C. Stevens et al., 11th Biennial Symp. Int. Assoc. Res. EBV Associated Dis., abstr. 13.02, 2004). Finally, we investigated the relationship between EBV DNA loads in blood and quantitative anti-EBV IgA and IgG serology in the Indonesian cohort. Previous studies predominantly compared the diagnostic performance of EBV DNA load measurement versus serological assays (47), but the putative quantitative relation between these two parameters has not been described.

MATERIALS AND METHODS

Patients. During the period from 2001 to 2003, 149 NPC patients were identified at the Department of Pathology, Gadjah Mada University, School of Medicine/Sardjito Academic Hospital (Yogyakarta, Indonesia). NPC diagnosis was based on pathological assessment of paraffin-embedded tumor biopsy specimens, EBER1/2 RNA in situ hybridization, and immunohistochemical staining for EBNA1 and LMP1 by using previously defined monoclonal antibodies (35, 36, 68). TNM staging for tumor size (T), lymph node involvement (N), and metastasis (M) was done by using the 1997 criteria of the Union International Contre le Cancer (49) for all patients by using clinical measurements and computer tomography scans as part of the routine patient workup. Approval of the local medical ethical committee was obtained.

Unfractionated whole-blood samples. Unfractionated EDTA-anticoagulated whole-blood samples were obtained from the NPC patients at the time of diagnosis and before any therapeutic intervention during their visit to the Sardjito Academic Hospital, Yogyakarta, Indonesia. Fresh EDTA-anticoagulated blood (0.5 ml) was directly lysed in 4.5 ml of NucliSens lysis buffer

(BioMerieux, Boxtel, The Netherlands) within 1 h after donation, mixed thoroughly, and immediately stored at enables short-term stabilization of nucleic acids at room temperature and long- term cryopreservation at -80oC (6, 11)

EBV nucleic acid isolation. EBV DNA and RNA were simultaneously isolated from 1 ml of lysed whole blood by silica-based extraction (4). Nucleic acids were eluted in 100 μl, and the nucleic acid equvalent form 5 μl of whole blood were used in subsequent DNA and RNA amplification assays. All reagents for nucleic acid isolation were obtained from BioMerieux.

EBV DNA load quantification by quantitative LC-based real-time PCR. The EBV DNA load in whole blood was determined by a quantitative LightCycler (LC) real-time PCR that targets a highly conserved 213-bp region of EBNA1. The primers used in this assay were QP1 and QP2, and the fluorigenic internal hybridization probes were EBNA LCN and EBNA FLN (TIBMolBiol, Berlin, Germany), described in detail elsewhere (53, 54). In addition, a newly developed LC-based PCR that amplifies a 99-bp part of EBNA1 (located within the 213-bp QP1-QP2 amplicon) was used for quantification of small DNA fragments. The experimental conditions, reagents, and hybridization probes in this 99-bp LC-based PCR assay were identical to those used in the 213-bp LC-based PCR assay, except that different primers were used (forward primer QP3 [5'-CCACAATGTCGTCTTACACC-3'] and reverse primer QP4 [5'ATAACAGACAATGGACTCCCT-3]). Real-time PCR reagents were obtained from Roche Diagnostics (Almere, The Netherlands). Tenfold dilutions of spectrophotometrically quantified plasmid DNA containing the EBNA1 target sequence were used to create a standard curve (53, 54). To check for putative inhibition of PCR, EBV DNA-negative samples were spiked with 1,000 copies of EBV plasmid DNA (53, 57). β-globin PCR was performed with primers PCO3 (5'-ACACAACTGTGTTCACTAGC-3') and PCO5 (5'-GAAACCCAAGAGTCTTCTCT-3'), which generate a 209-bp PCR product. Reaction conditions were as follows: a volume of 50 μ l with 50 mM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, 200 M deoxynucleoside triphosphate, 10 mM Tris (pH 8.5), and 1 U of *Taq* polymerase (Perkin-Elmer). Amplification was done for 40 cycles of 1 min at 95°C, 2 min at 55°C, and 1.5 min at 72°C. The first denaturation step and the last elongation step were extended for 4 min. PCR products were visualized by standard agarose gel electrophoresis.

NASBA for EBV RNA detection. Nucleic acid sequence-based amplification (NASBA) assays for BARF1 and EBNA1 mRNA and the low-copy-number human U1A small nuclear ribonucleoprotein (U1A snRNP) housekeeping mRNA were done as previously described by us (5, 19, 21). NASBA is an isothermal RNA amplification technique that enables highly specific RNA amplification in a DNA background, regardless of the splice patterns (13). NASBA reagents were obtained from BioMerieux (NucliSens basic kit). The primers for BARF1 and EBNA1 were located within the open reading frames of these genes, which enabled simultaneous amplification of all putative splice variants (5, 21). Both assays yield amplification products of 203 nucleotides. The marmoset lymphoblastoid cell line B95-8, which expresses both EBNA1 and BARF1 mRNA transcripts, was used as a positive control. The sensitivities of the BARF1 and EBNA1 NASBAs were previously determined to be 10 mRNA molecules or <1 EBV-positive RNA cell equivalent (5).

Several precautions described previously (29) were taken during the PCR and NASBA procedures to avoid false positivity. In all experiments appropriate negative and positive controls were included during nucleic acid isolation and amplification.

EBV serology. IgG seroreactivity against EBV was assessed by immunoblotting by using the nuclear fraction of HH514.c16 cells, which were chemically induced to express the viral capsid antigen (15). Immunoblots were scored semiquantitatively from 1 (weakest) to 4 (strongest), with reference to controls analyzed in parallel (15). IgA reactivity was quantitatively assessed by a

synthetic peptide-based enzyme-linked immunosorbent assay (ELISA) for immunodominant epitopes derived from EBNA1 and VCA-p18 (BFRF3), as described previously (15).

RESULTS

EBV DNA load in whole-blood samples of NPC patients determined by 213-bp EBNA1 LC-based PCR. Initially, we used a quantitative LC-based real-time PCR assay that targets a 213-bp region of EBNA1 for determination of the EBV DNA loads in unfractionated blood samples. Of 149 fresh whole-blood samples obtained from 149 NPC patients, 108 (72.5%) were positive by this 213-bp LC-based PCR, with EBV DNA loads ranging from 0 to 110,400 copies/ml blood (mean, 4,167 EBV DNA copies/ml blood; median, 1,050 EBV DNA copies/ml blood). Forty-four samples (29.5%) had loads above the 2,000-copy/ml cutoff value. This value of 2,000 copies/ml corresponds to 10 EBV DNA copies per PCR tube, based on a DNA input in PCR equivalent to 5 l unfractionated whole blood (54, 55). Ten copies per reaction is the lower limit of the EBV DNA load that can still be reliably quantified, albeit with a large standard deviation (54). Lower amounts of EBV DNA per reaction may provide a positive result but cannot be reliably quantified by real-time PCR.

All EBV DNA-negative samples were positive by the β-globin PCR. Spiking of EBV DNA-negative samples with small amounts of EBV plasmid DNA in quantitative LC-based PCR revealed that the LC-based PCR-negative samples were not inhibited for DNA amplification but were truly EBV DNA negative.

LC-based PCR target size significantly influences the EBV DNA load in blood of NPC patients. We reasoned that if the EBV DNA in whole blood was (partially) fragmented, e.g., due to apoptosis, which yields fragments of approximately 150 bp in length (39), the use of a smaller DNA target size in the real-time PCR would give a higher EBV DNA load. Therefore, we developed a novel 99-bp LC-based real-time PCR that targets EBNA1 with QP3 and QP4 primers located internally in the 213-bp QP1-QP2 amplicon (53, 54). This enabled the use of the same validated set of hybridization probes and the same standard dilution curve of EBNA1 plasmid DNA in both PCR assays. The 99-bp LC-based PCR had an amplification efficiency similar to that of the 213-bp LC-based PCR assay, based on the slopes of the amplification curves in the log-linear phase for the individual clinical samples and for the standard dilution samples consisting of EBNA1 plasmid DNA. The mean amplification efficiencies were calculated for both the 99-bp and the 213-bp LC-based PCRs by using the standard curve data for each run (n=10 runs per LC-based PCR assay). The mean amplification efficiency in the 213-bp PCR was 96.2%, with a standard deviation of 2.6%. In the 99-bp LC-based PCR assay, the mean efficiency was 95.3%, with a standard deviation of 2.7%. There was no statistically significant difference between the amplification efficiencies in the two LC-based PCR assays (Mann-Whitney test, p=0.367). Furthermore, both assays gave the same quantified DNA amount at different inputs of spiked EBNA1 plasmid DNA; these amounts ranged from 10 to 10^5 copies.

The number of whole-blood samples from NPC patients positive for EBV DNA by the 99-bp LC-based PCR assay was 128 of 149 (85.9%; compared to 72.5% in the 213-bp PCR). The range was 0 to 8,962,000 EBV DNA copies/ml blood (median, 4,048 EBV DNA copies/ml blood; mean, 86,960 EBV DNA copies/ml blood). Ninety of 149 samples (60.4%) had loads above the 2,000-copy/ml cutoff value (compared to 29.5% by the 213-bp PCR). Eight samples had loads copies/ml by the 99-bp LC-based PCR but were negative by the 213-bp LC-based PCR. The 99-bp LC-based PCR assay quantified a statistically significantly higher EBV DNA load in whole-blood specimens than the 213-bp LC-based PCR assay (Wilcoxon's test, P < 0.0001) (Fig. 1). The mean

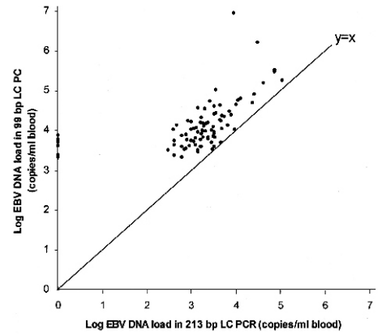

Fig. 1. Comparison of EBV DNA loads in blood of NPC patients as determined by the 213-bp and 99-bp EBV LC-based real-time PCR. All 90 samples with EBV DNA loads 99-bp LC-based PCR are included (corresponding to LC-based PCR, the lower limit that can still be reproducibly quantified). The 99-bp LC-based PCR assay significantly quantifies a higher EBV DNA load than the 213-bp LC-based PCR assay (Wilcoxon's test, P=0.0001). The line y x denotes equal amounts of EBV DNA in both assays.

difference was a 19.7 times higher EBV DNA load by the 99-bp LC-based PCR than by the 213-bp LC-based PCR assay (median difference, 4.9 times; range, 1.1 to 995.8 times).

The increase in the EBV DNA load by the 99-bp PCR compared to that determined by the 213-bp PCR was highly variable between individual whole-blood samples and was not related to the absolute EBV DNA amount in the sample in either assay (Fig. 1). Our results show that the EBV DNA load is highly dependent on the PCR target size and are consistent with the data obtained by Chan et al. (8), which indicated that the viral DNA circulating in NPC patients is fragmented.

Fourteen healthy EBV-seropositive donors were tested for EBV DNA positivity by the 99-bp LC-based PCR assay. All were negative, confirming previous studies showing low cell-associated EBV DNA loads in the blood of healthy EBV carriers, with approximately only 1 to 10 per 10^6 circulating B cells carrying the EBV genome (54, 55, 63, 67).

Relation between circulating EBV DNA positivity and PCR amplicon length. The proportion of NPC patients positive for EBV DNA in their circulation has been remarkably variable between studies published to date and ranges from 30% to 98% (9, 24, 25, 31, 38, 47). Based on the indications for fragmented EBV DNA (8; see above), we tried to find a possible explanation for this wide range of positivity and differences in clinical sensitivity. Therefore, we investigated the relationship between PCR amplicon length and circulating EBV DNA positivity in patients at the time of primary diagnosis of NPC using data from our present study and nine previous studies with sufficient information on the DNA target size used in PCR and the patient positivity rate. As is shown in Fig. 2, there is a statistically significant inverse correlation between the reported number of patients positive for EBV DNA in their circulations and the length of the amplicon in PCR (Spearman's rho = 0.91 P < 0.0001), independent of the PCR technique, EBV target sequence, or clinical specimen type.

Relation of TNM staging and EBV DNA load in blood of NPC patients. We hypothesized that advanced NPC stages or larger tumors would be related to higher EBV DNA loads in blood. However, we did not find a relation between the TNM stage of the primary tumor and the presence or the level of EBV DNA in the circulation, as determined by either the 213-bp LC-based PCR (Kruskal-Wallis test, P=0.423) or the 99-bp LC-based PCR (P 0.658). Furthermore, the

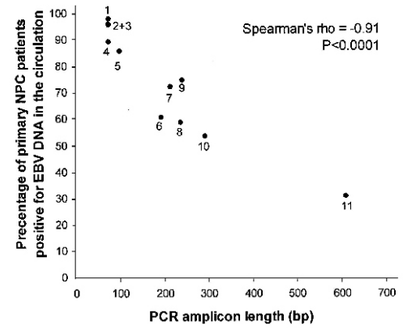

Fig. 2. Correlation between PCR amplicon lengths and percentage of patients with a primary diagnosis of NPC positive for EBV DNA in their circulation, using data from 11 different studies. The data for the following points are from the indicated references: 1 (31), 2 (47), 3 (34), 4 (7), 5 (this study), 6 (9), 7 (this study), 8 (48), 9 (25), 10 (24), and 11 (38).

primary tumor size (T1 to T4) did not correlate with the circulating EBV DNA load by either the 213-bp LC-based PCR (Kruskal-Wallis test, P=0.260) or the 99-bp LC-based PCR (P=0.218).

Absence of BARF1 mRNA expression in the circulation of NPC patients indicates a virtual absence of intact circulating NPC cells. To investigate whether (part of the) EBV DNA in NPC patients was derived from intact circulating tumor cells and whether detection of the carcinoma-specific, EBV-encoded BARF1 mRNA in the circulation of NPC patients has diagnostic value, we performed NASBA for this oncogene using whole-blood RNA obtained from 19 NPC patients with the highest EBV DNA loads in both the 99-bp LC-based PCR (range, 4,048 to 8,962,000 EBV DNA copies/ml blood) and the 213-bp LC-based PCR (range, 2,600 to 75,200 EBV DNA copies/ml blood). All 19 samples were positive for low-copy-number human housekeeping gene U1A snRNP RNA, indicating amplifiable RNA quality. Seven of 19 samples (37%) were EBNA1 mRNA positive, but none was BARF1 mRNA positive. To exclude the possibility that the absence of BARF1 mRNA in whole blood was due to the absence of this transcript in the primary tumor itself, we investigated whether this mRNA was detectable in nasopharyngeal brush specimens obtained simultaneously with the whole-blood samples. Brush specimens were available from 13 of the 19 patients indicated above. In 12 of 13 cases, BARF1 mRNA was clearly detectable, and extremely elevated EBV DNA loads were found (>15,000 copies/brush sample) in all brush specimen except the BARF1 mRNA-negative brush sample, which had a load below 2,000 EBV DNA copies/brush sample (S. J. C. Stevens et al., unpublished data; Stevens et al., 11th Biennial Symp. Int. Assoc. Res. EBV Associated Dis.).

Correlation of EBV DNA loads in whole blood with EBV-specific IgG and IgA serology. The molecular diversity and intensity of serological responses against EBV proteins in the cohort of Indonesian NPC patients were recently described by us, and serological data were available for 139 of 149 patients (15). We correlated the semiquantitative immunoblot score (score of 1 to 4) to the whole-blood EBV DNA load, as determined by the 99-bp or the 213-bp LC-based PCR. As shown in Fig. 3A and B, no significant difference was observed between these four groups (Kruskal-Wallis test, P=0.124 for the 99-bp LC-based PCR and P 0.091 for the 213-bp assay). Furthermore, the EBV DNA load in blood did not correlate significantly with anti-EBNA1 IgA levels

(Spearman's rho= -0.14 and -0.113 for the 213- and 99-bp LC-based PCRs, respectively) (Fig. 4A and B) or with anti-VCA p18 IgA levels (Spearman's rho = -0.102 and -0.099 for the 213-bp and 99-bp LC-based PCRs, respectively) (Fig. 4C and D). Finally, ELISA optical density (OD) values for anti-EBNA1 IgA did not differ significantly between EBV DNA-positive and -negative whole-blood samples by either the 99-bp or the 213-bp LC-based PCR (Mann-Whitney test, P =0.115 and 0.955, respectively), nor did the OD values in the anti-VCA-p18 IgA ELISA differ (P =0.814 and 0.103, respectively). Thus, circulating EBV DNA and EBV antibody responses are independent and quantitatively unrelated parameters in individual NPC patients.

DISCUSSION

This study is the first to investigate the primary diagnostic value of circulating EBV DNA load determination and its relation to quantitative EBV serology in Indonesian NPC patients. In general, the significantly higher whole-blood EBV DNA loads in these NPC patients compared to

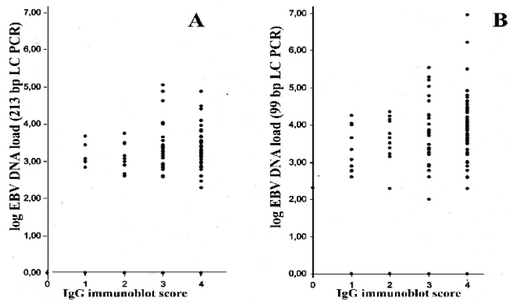

Fig. 3. EBV DNA load in blood of Indonesian NPC patients is not related to anti-EBV IgG reactivity, as assessed by immunoblotting. EBV-specific immunoblot data and scoring were previously described by us (15). The overall immunoblot intensity was scored semiquantitatively as negative (n=1, group 0), 1 (n=12, group 1), 2 (n=16, group 2), 3 (n=33, group 3), and 4 (n=77, group 4). No significant difference in EBV DNA load was observed between the four groups for either the 213-bp LC-based PCR (A)(Kruskal-Wallis test, P=0.091) or the 99-bp LC-based PCR (B) (P=0.124).

those in healthy EBV carriers (43, 55, 56, 63) confirm the findings for Chinese, Thai, Italian, and Taiwanese NPC patients (10, 38, 41, 47, 48, 60, 66). Still, about 15% of the whole-blood samples from our patients were EBV DNA negative, while the load in an even larger fraction (40%) was below the 2,000-copy/ml clinical cut off value previously determined to be the upper limit in healthy EBV carriers (54, 55). This is in agreement with previous studies showing the absence of plasma or serum EBV DNA in a considerable proportion (15 to 50%) of NPC patients at the time of primary diagnosis and the corresponding low clinical sensitivities (90%) of EBV DNA load monitoring (9, 24, 25, 38, 48). These observations question the general use of EBV DNA load monitoring for the primary or confirmatory diagnosis of NPC in future population-based screening studies. In contrast, studies in Hong Kong by Lo and co-workers, predominantly by a PCR that targeted the (variable) BamHI-W repeat domain, invariably showed the high specificity of plasma EBV load quantification, although only trace amounts of EBV DNA (were found in some NPC patients, while healthy blood donors are also occasionally positive (7, 18, 30, 31).

Our study now explains the large variations in the diagnostic sensitivity of circulating EBV DNA load quantification reported in the literature. As the majority of EBV DNA may be fragmented,

a small PCR target size will quantify a significantly higher EBV load (Fig. 1) and yield more EBV DNA-positive samples (Fig. 2), which we show is independent of the PCR technique, the amplicon, or the clinical specimen used. These findings also substantiate a recent, smaller study that found that the majority of plasma EBV DNA in NPC patients is highly fragmented (180 bp) and is not virion associated (8). It probably originates from the release of the DNA from apoptosed tumor cells into the circulation (8). However, Shotelersuk et al. (48) found DNase-resistant circulating DNA in more than 50% of NPC patients tested, suggesting that EBV DNA is protected in virions. In contrast to those two studies (8, 48), which both used plasma for EBV DNA characterization, we used fresh whole blood that was directly lysed in a nucleic acid-stabilizing buffer within 1 h after collection. This approach enables parallel EBV DNA and RNA analyses; ensures a direct fixation of the *in vivo*

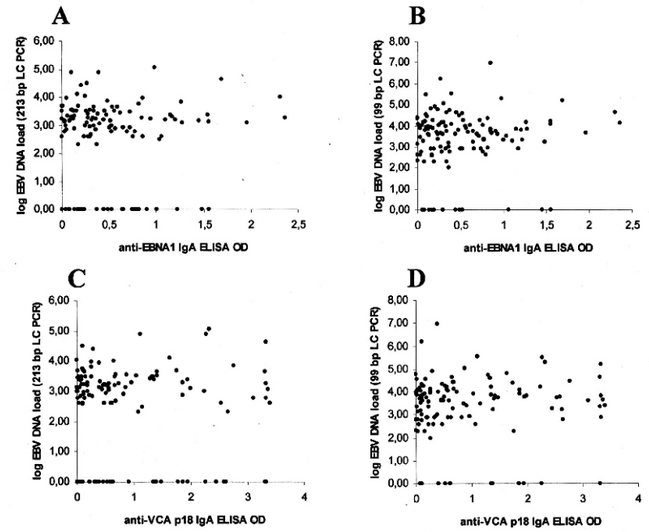

Fig. 4. Circulating EBV DNA loads in Indonesian NPC patients do not correlate with quantitative EBV IgA serology, as determined by synthetic peptide-based ELISA for immunodominant epitopes of EBNA1 and VCA-p18. (A and B) Relation between anti-EBNA1 IgA ELISA OD values (with the background subtracted) and EBV DNA loads in whole blood determined by the 213-bp or the 99-bp LC-based PCR (Spearman's rho = -0.14 and -0.113 respectively), (C and D) relation for anti-VCA-p18 ELISA OD values (Spearman's rho = -0.102 and -0.099 for 213-bp and 99-bp LC-based PCRs, respectively)

EBV DNA and RNA status; and may increase the sensitivity by combining plasma and cellular blood compartments, which may both harbor EBV DNA (32, 41, 57). The use of whole blood omits cell separation techniques and delayed preparation of plasma, factors that may possibly affect viral DNA and RNA characteristics by inducing uncontrollable cell lysis, apoptosis, or lytic virus replication (52).

The absence of circulating BARF1 mRNA, despite high EBV DNA loads, further substantiates the fact that the circulating EBV DNA is probably not derived directly from intact circulating NPC cells. The undetectable presence of only small numbers of circulating NPC cells which have silenced their BARF1 RNA expression cannot be excluded. This then, however, would

be selective for circulating cells because BARF1 mRNA can easily be detected in NPC tissue biopsy and brush specimens (5, 21). There is also a possibility of quick in vivo RNA degradation upon the immediate lysis of tumor cells entering the circulation.

The detection of EBNA1 mRNA in the circulation may reflect increasing numbers of EBV-positive B cells, as already suggested in a previous study that showed elevated EBV DNA loads in peripheral blood mononuclear cells of Taiwanese NPC patients (32). This phenomenon may relate to de novo B-cell infection due to local lytic viral replication in NPC (16, 61, 68), of which IgG and IgA reactivity to numerous early and late lytic viral antigens in NPC patients is a reflection (15). An enhanced replication frequency of circulating EBV-positive B cells may lead to higher levels of EBNA1 mRNA expression (14, 23). Furthermore, EBNA1 transcription in blood indicates that not all EBV DNA is fragmented but that a part of the circulating viral DNA load is cell associated in the context of an intact and transcriptionally active EBV genome (23), in agreement with the findings of Lin et al. (32) and Shotelersuk et al. (48).

The indications for EBV DNA fragmentation may have profound diagnostic consequences. The PCR target size should preferably be as small as technically possible to increase detection rates and to assess the EBV load as accurately as possible. Still, a recent study that used a 72-bp PCR found a low sensitivity (0.85) similar to that found in our present study at a diagnostic cutoff of 7 copies/ml serum (28). We consider such a low value to be diagnostically irrelevant. Intrinsic to real-time PCR assays, small amounts of DNA (100 copies/reaction) cannot be as accurately and reproducibly quantified as large amounts (54, 63).

The fragmentation of viral DNA was recently also reported for another herpesvirus, i.e., cytomegalovirus, in the blood of transplant recipients (3), a population in which monitoring of circulating EBV DNA loads is also widely applied. Discussions about the sources and characteristics of circulating EBV DNA and the preferred clinical specimen type in different patient populations are ongoing (20, 44, 56, 62) and should also quantitatively address the origin and the physical nature of the EBV DNA. In NPC patients this should be extended to defining a lymphoid versus an epithelial cell tropism of EBV. It may, however, be technically challenging to quantitatively detect and differentiate EBV DNA originating from lysed NPC cells, circulating intact tumor cells, latently infected B cells, and/or virions.

Despite the development of a short-fragment LC-based PCR and the fact that the majority of patients had NPC stage III or IV disease, circulating EBV DNA load quantification was not sensitive enough for primary diagnosis either in this study or in numerous other studies from regions where NPC is endemic (24, 28, 31, 38, 41, 48). Thus, EBV DNA load measurement may have limited value for the primary or confirmatory diagnosis of NPC in population screening in high-incidence regions and is not recommended as the sole means of risk assessment. Finally, our results demonstrate that serology and the EBV DNA load in blood are independent parameters that are not quantitatively related to each other. This may explain why the combination of circulating EBV DNA detection and EBV serology increases the diagnostic sensitivity (9). By using follow-up sampling, we are studying whether monitoring of EBV DNA parameters in whole blood is useful for the prediction of therapeutic efficacy and NPC recurrence or persistent disease in patients with elevated viral DNA loads at the time of primary diagnosis (30, 33).

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CHAPTER 7

Noninvasive Diagnosis of Nasopharyngeal Carcinoma: Nasopharyngeal Brushings Reveal High Epstein-Barr virus DNA Load and Carcinoma-Specific Viral BARF1 mRNA

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is the most prevalent ENT tumour in Indonesia. We investigated the primary diagnostic value of Epstein-Barr virus (EBV) DNA load and mRNA detection in noninvasive nasopharyngeal (NP) brushings, obtained prospectively from consecutive Indonesian ENT-patients with suspected NPC (n=106) and controls. A subsequent routine NP biopsy was taken for pathological examination and EBER-RISH, yielding 85 confirmed NPC and 21 non-NPC tumour patients. EBV DNA and human DNA load were quantified by real-time PCR. NP brushings from NPC patients contained extremely high EBV DNA loads compared to the 88 non-NPC controls ($p < 0.0001$). Using mean EBV DNA load in controls plus 3 SD as cut-off value, specificity, sensitivity, positive and negative predictive values were 98, 90, 97 and 91%, respectively. Epstein-Barr nuclear antigen 1 (EBNA1) and the carcinoma-specific BARF1 mRNA were detected by nucleic acid sequence based amplification and found in 86 and 74% of NP brushings, confirming NPC tumour cell presence. EBV RNA positivity was even higher in fresh samples stored at -80^{o}C until RNA expression analyses (88% for both EBNA1 and BARF1). EBV RNA-negative NP brushings from proven NPC cases had the lowest EBV DNA loads, indicating erroneous sampling. No EBV mRNA was detected in NP brushings from healthy donors and non-NPC patients. In conclusion, EBV DNA load measurement combined with detection of BARF1 mRNA in simple NP brushings allows noninvasive NPC diagnosis. It reflects carcinoma- specific EBV involvement at the anatomical site of tumour development and reduces the need for invasive biopsies. This procedure may be useful for confirmatory diagnosis in large serological NPC screening programs and has potential as prognostic tool.

INTRODUCTION

Undifferentiated nasopharyngeal carcinoma (NPC WHO type III) is virtually 100% associated with Epstein-Barr virus (EBV) and has a reported high incidence in most of South-East Asia and intermediate incidence in North-African populations and in Inuit (13). In Indonesia, with an ethnically diverse population of 225 million people, NPC is the most common ENT tumour with high prevalence among native populations and a yearly overall incidence estimated at 6.2/100,000 (4). Extremely high incidence was recently documented in native populations living on the island of Sulawesi (5). In Yogyakarta, Central Java, NPC is the most prevalent tumour among man and 4th most prevalent among females, with a male/female ratio of 2.4, constituting respectively 22 and 8% of all diagnosed malignancies (4).

The strong etiological link between EBV and NPC has been known for over 3 decades (13, 6) and is reflected by abnormal anti- EBV antibody profiles, increased circulating EBV DNA levels and by distinct EBV gene expression in the tumour cells (711). Classically, NPC is considered to have a latency type 2 EBV transcription, with expression of EBV-encoded small RNAs 1 and 2 (EBER1/2), BamHI A rightward transcripts (BARTs), Epstein-Barr nuclear antigen 1 (EBNA1) and latent membrane protein 2 (LMP2), while LMP1 is more heterogeneously expressed (2, 12, 13) Previously, we and others showed additional transcription of a viral oncogene encoded in the BamHI-A rightward frame 1 (BARF1).10,14,15 BARF1 mRNA is exclusively expressed in EBV positive carcinomas (i.e. NPC and EBV-positive gastric carcinomas) and is virtually absent from EBV-linked lymphoma (10, 15). BARF1 has transforming activity in vitro and in vivo (1618). BARF1 encodes a 33 kDa type-II membrane protein that can be cleaved after amino acid 20 to release a soluble 29 kDa fragment with mitogenic activity, whereas the remaining transmembrane domain increases bcl-2 expression, thus contributing to cell growth and survival. Recent reports further substantiated that BARF1 protein is rapidly and efficiently secreted by epithelial cells (1921).

Currently, diagnosis of WHO type III NPC requires a biopsy from the primary tumour site or metastases for histopathological assessment and demonstration of EBV involvement by in situ hybridization for EBER1/2. Even at the early stages of NPC, patients are characterised by aberrant serological responses to EBV compared to healthy EBV-carriers. Both IgG and IgA antibody responses to defined EBV proteins may be used as NPC markers (11). Quantitation of circulating EBV DNA may be useful for prognostic monitoring in a subset of patients (9), but because of low or negative EBV DNA values in a significant number of NPC patients, this method is less suited for primary diagnosis (22). EBV DNA in the blood of NPC patients appears highly fragmented, reflecting tumour apoptosis or necrosis (22, 23).

Because serology and quantitation of circulating viral DNA only indirectly reflect carcinoma-associated activity of EBV in the nasopharyngeal (NP) region, we further explored the consistent etiological link between NPC and EBV in this study. We hypothesized that NPC may be more directly reflected by elevated viral DNA levels plus carcinoma-specific viral transcriptional activity at the site of the primary tumour. This is indicated by recent reports, showing elevated EBV DNA loads in NP swab samples of NPC patients using nonstandardized PCR techniques (2427). Thus, we investigated in more detail the diagnostic value of EBV DNA load quantification and EBV mRNA detection, in particular BARF1 and EBNA1 mRNA, in NP brushing specimens from consecutive Indonesian ENT patients with suspected NPC and various controls. In all patients suspected for NPC a biopsy was taken from the same site to confirm presence or absence of NPC by using EBER in situ hybridisation (EBER-RISH) and immunohistochemical staining for EBNA1 and LMP1. We used a clinically well-validated LightCycler (LC)-based real-time PCR for rapid and accurate EBV DNA load determination (28, 29) and nucleic acid sequence based amplification (NASBA) assays for sensitive and specific detection of viral mRNAs in high EBV DNA backgrounds (10, 15). RT-PCR was used in addition to confirm the presence of spliced EBV mRNA. This combined EBV DNA/ mRNA approach may enable diagnosis of NPC by directly revealing aberrant tumour-related EBV activity at the anatomical site of NPC tumour development. This noninvasive procedure may allow objective NPC diagnosis and may reduce the number of invasive biopsies required. It may also be used as confirmatory test in serological screening programs and is a putative prognostic tool.

MATERIALS AND METHODS

Patients. NPC patients (n=85) were identified at Sardjito Academic Hospital, Gadjah Mada University School of Medicine, (Yogyakarta, Indonesia) in a population of 106 ENT-patients suspected of having NPC based on first clinical examination in the period 2002-2004. Diagnosis was based on pathological assessment of paraffin-embedded tumour biopsy specimens, EBER1/2 in situ hybridisation using commercial PNA-based hybridisation probes (Dakocytomation, Glostrup, Denmark) and immunohistochemical staining for EBNA1 and LMP1, using previously described monoclonal antibodies (30, 31). Approval of the local medical ethical committee was obtained for this study and all patients and controls signed for informed consent. TNM staging was done for all patients as described previously (32) using clinical measurements and CT scans as part of the routine work-up.

Cell lines. The EBV-positive NPC cell line C666-1 (kindly provided by Dr. Dolly Huang), the EBV-positive marmoset lymphoblastoid cell line (LCL) B95-8 and the human LCL JY were used as positive control for RNA amplification. Besides EBV latent RNAs, both LCLs express B-cell associated lytic cycle EBV RNAs in a minority of cells, including BARF1 mRNA. The C666-1 NPC cell line has a latent EBV transcription phenotype including BARF1 mRNA expression. The EBV-

negative Burkitt's lymphoma cell line Ramos (ATCC CRL-1596) was used as negative control.

Nasopharyngeal brushing samples. NP brushing was performed by experienced ENT-specialists and ENT resident trainees. In all cases, the NP brush sample was taken prior to the biopsy in patients with suspected NPC, and both were sampled from the same site, as defined by nasendoscopy. In total, 85 NP brush samples were obtained from patients with subsequently biopsy-proven EBER-positive NPC. Suspected NPC patients who yielded an EBER-negative biopsy but were diagnosed with ENT malignancy were included as non-NPC tumour controls. In total, 88 control NP brushings were sampled, obtained from 21 patients with non-EBV-associated head and neck carcinomas and 22 patients with other otorhinolaryngological complaints; as well as from healthy EBV-seropositive donors from the Yogyakarta region (n=28) and Amsterdam (n=15) and 2 EBV-seronegative donors.

Nasendoscopy-guided NP brushings were taken after applying local anaesthetic spray (1% Lidocaine; AstraZeneca, Waltham, MA). A flexible nasendoscope was used to evaluate the entire nasopharynx and the site of tumour involvement. For the brushing, a standard Cytobrush Plus (Medscand, Malm€o, Sweden) with a wire shaft was used, which was contained in a plastic catheter covering the entire shaft of the brush, to prevent contamination of cells from non-NP sites. The catheter with brush was inserted via the nose until the nasopharynx was reached. Then the brush was released from the catheter and the cytobrush was gently rotated for several times over the NP epithelium, returned into the catheter and removed. Subsequently, for an initial series of samples, 2 smears were made on glass slides for cytological evaluation and then the cytobrush tip (~1.5 cm) was cut-off and placed in 4 ml of NucliSens lysis buffer (BioMerieux, Boxtel, The Netherlands), mixed well and stored at -80^{0}C until use. This buffer instantly stabilizes DNA and RNA, permitting short-term transport and storage at ambient temperatures and enabling long-term preservation at -80^{0}C (33, 34).

EBV parameters in the brushing specimens were retrospectively analysed batch wise and in a blinded fashion at the department of Pathology, VU Medical Centre, Amsterdam, The Netherlands, uninformed about the NPC status of the patients. For this purpose NP brushing samples collected in Indonesia and stored in lysis buffer within 2 hr after collection were sent on dry ice to Amsterdam in 2 batches. The first arrived in thawed condition, whereas the second was kept on dry ice during the entire transport. Furthermore, in order to yield as many NPC cells as possible for DNA and RNA analyses, brushes from the second batch were directly put in NucliSens lysis buffer without preparing glass slides for cytology. This was also done for the posttherapy follow-up samples.

Blood and serum samples. Blood samples (9 ml) were drawn at the time of NP brushing/biopsy collection and 0.5 ml was mixed immediately with 4.5 ml NucliSens lysis buffer and stored at -80^{0}C. The remaining blood was used for preparation of serum by clotting at 4^{0}C overnight and subsequent removal of the clot by centrifugation. Serum was aliquoted and stored at -20^{0}C.

Nucleic acid isolation by silica-based extraction. DNA and RNA were simultaneously isolated from NP brushing samples by silica-based nucleic acid extraction as described previously (35). One milliliter of lysate was used as input for the isolation procedure and the nucleic acids were eluted in 100 µl of elution buffer. Reagents for the isolation procedure were obtained from BioMerieux, Boxtel, The Netherlands.

EBV DNA load and cellular DNA quantification by quantitative LC-based real-time PCR assays. EBV DNA load in NP brushings was determined by a quantitative LC-based real-time PCR targeting a highly conserved 213 bp region of EBNA1, a single copy gene of EBV. This assay was described in detail by us elsewhere (28, 29). The amount of human diploid genome equivalents

in NP brushing specimens was determined by quantitative LC-PCR targeting a 197 bp fragment of the human β-globin gene (36). Five microliters of nucleic acid eluate was used as input for all PCR assays. b-globin DNA-negative samples (n=1) were excluded from the study.

Nucleic acid sequence based amplification for EBV RNA detection. NASBA is a sensitive, isothermal RNA amplification technique that enables specific RNA amplification in a high DNA background, regardless of RNA splice patterns (37). NASBA reagents were obtained from BioMerieux (NucliSens basic kit, BioMerieux, Boxtel, The Netherlands). Oligonucleotide primers for LMP2 and (nonspliced) BARF1 mRNA were described before (10, 15). EBNA1 NASBA primers were located within the (nonspliced) open reading frame of this gene, enabling simultaneous amplification of all putative splice variants and EBNA1 transcripts derived from C, W, Q or F promoter (10,15). Analytical and relative sensitivities of the NASBA assays were previously determined at (10). mRNA molecules and an RNA amount equivalent to <1 EBV-infected cell, respectively (10). Finally, for detection of A3/A4-spliced BART RNA we used an RT-PCR assay (38). Several precautions were taken during PCR and NASBA to avoid false positivity, as described previously (29). In all experiments appropriate negative and positive controls were included during nucleic acid isolation and amplification.

EBV serology. All ENT patients and healthy controls were analysed for serum IgG and IgA antibodies to EBV-specific and immunodominant VCA-P18 (BFRF3) and EBNA1 (BKRF1) epitopes using synthetic peptide-based ELISA tests and by immunoblot for EBVspecific IgG and IgA antibody-diversity profiling, exactly as described before (11).

Healthy controls were characterised by restricted IgG antibody diversity profiles with IgG antibodies to VCA-p18 and/or EBNA1, but rarely had EBV-specific IgA antibodies. NPC patients were characterised by positive IgG and IgA responses to VCA-p18 and EBNA1 epitopes and all had an abnormal IgG diversity profile on immunoblot, used as confirmatory test (1).

RESULTS

NP brushings of NPC patients contain extremely high EBV DNA loads. The NP brushing procedure was well tolerated and none of the patients or controls complained of any negative effects caused by the brushing procedure, except for nasal dripping or relative mild bleeding in ~ 40% of suspected NPC patients and an occasional healthy control, which was nowhere comparable to the excessive bleeding and pain often observed during and after taking the NP biopsy. The use of a flexible endoscope facilitated proper sampling at the site of suspected tumour mass.

To assess whether the brushing procedure was a reproducible approach to collect cells from the nasopharynx, the amount of human diploid genome equivalents was determined first by LC-PCR in 61 NP brushing samples (48 NPC patients and 13 controls). This yielded a highly consistent and comparable value for NP brushings of both NPC patients and controls (mean 5.3 X 10^6; range 4.8 X 10^5 2.9 X 10^7), indicating that the NP brush procedure is a reliable and reproducible means of sampling (Fig. 1). No difference was observed for the amount of human diploid genome equivalents in NP brushings from NPC patients vs. controls (Mann-Whitney test, p 5 0.21).

Quantitative analysis of EBV DNA copies was performed using a standardised and well-validated LC-based real-time PCR for the single copy EBNA1 gene. All NP brushing samples (100%) from NPC patients (n=85) were positive for EBV DNA, with extremely high viral DNA loads (median: 2.37 3 10^6 EBV DNA copies/brushing; mean: 5.45 X 10^6 EBV DNA copies/brushing; range 1.08 X 10^2 4.88 X 10^7; Fig. 2).

In the NP brushings from the control population (n=88 in total), EBV DNA was detectable in 68/88 cases (77%) but the EBV DNA load was very low ranging from 04, 158 EBV DNA copies/NP

brushing only (median: 177; mean: 376 EBV DNA copies/NP brushing). A statistically significant higher EBV DNA load was observed in NPC patients compared to controls (Mann-Whitney test p < 0.0001), with only marginal overlap between these 2 groups, as shown in Figure 2. In the controls, consisting of 21 non-NPC tumour patients, 22 patients with other ENT-complaints and 45 healthy

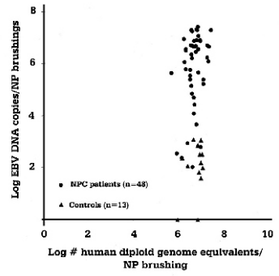

Fig 1. NPC patients and controls have similar numbers of human diploid genome equivalents/NP brushing, as determined by quantitative LightCycler-based real-time PCR and EBV DNA load in nasopharyngeal brushing samples ("EBV DNA copies/NP brushing") is unrelated to the number of diploid genome equivalents.

donors, no differences were observed in the mean values of EBV DNA loads between the different populations, which were similarly low in all groups (Fig. 2).

Cut-off values (COV) for EBV LC were defined by calculating the mean EBV DNA load in NP brushings from the control population plus 2 standard deviations (i.e. 1666 EBV DNA copies/NP brushing) or the mean plus 3 standard deviations (i.e. 2312 EBV DNA copies/NP brushing). These COV were used to determine sensitivity, specificity, positive and negative predictive values, as indicated in Table I. Using COV, these diagnostic parameters were ~90%. Of the 79 individuals exceeding the COV in PCR, 77 were confirmed as NPC cases by EBER-positive biopsies (Table I). Only 2 controls (1 healthy EBV-carrier and 1 non-NPC tumour patient) showed a slightly elevated EBV DNA load above COV, illustrating the high specificity of this approach (Fig. 2, Table I). Interestingly, the healthy carrier showed IgA reactivity to EBNA1 and VCA-p18, which is very characteristic for NPC patients, but is normally rarely observed in healthy EBV-carriers, 11 indicating putative aberrant EBV activity in this individual. We are now regularly examining this volunteer for EBV and clinical parameters.

The EBV DNA load in the NP brush samples from NPC patients showed no relation to the number of cells, defining 1 cell as 2 diploid human genome equivalents (Spearman's ñ = 0.29; see Fig. 3), indicating that most DNA collected on the brush was derived from normal cells. This is in agreement with the cytological observation revealing many polymorph nuclear leukocytes and normal columnar epithelial cells with only sporadic NPC tumour cells per field of microscopic observation (data not shown). Two EBV-negative healthy individuals yielded negative EBV DNA values whereas the cellular DNA load was within the normal range. A significant linear correlation was obtained between the EBV DNA load expressed per NP brush and the EBV DNA load expressed per human diploid genome equivalent (Spearman's $\rho = 0.94$; $p < 0.0001$), as shown in Figure 3. This reflects the constant level and abundance of normal cells relative to NPC cells in the brush samples. Therefore, the value of EBV DNA load per brush was used for all further analyses. The amount of EBV DNA in the NP brushing was not related to TNM stage (Kruskal-Wallis test p = 0.223) or primary tumour size (Kruskal-Wallis test p = 0.755). Finally, EBV DNA load in the NP

brushing was unrelated to EBV DNA load in the whole blood of patients ($r^2 = 0.05$; p 5 0.07), as determined by a recently described 99 bp LC-PCR (22).

EBV RNA is abundantly detectable in NP brushings: EBV is transcriptionally active

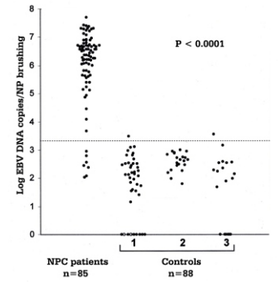

Fig. 2. Nasopharyngeal brushings obtained from NPC patients have significantly higher EBV DNA loads compared to non-NPC controls (p < 0.0001). Control group 1 consists of 45 healthy individuals (open circles indicate the 2 EBV-seronegative individuals), control group 2 of 22 patients with ENT complaints but without malignancy and control group 3 consists of 21 non-NPC tumour patients. The dotted line indicates the cut-off value, calculated as the mean EBV DNA load in NP brushings from the controls plus 3 standard deviations.

and expresses the carcinoma-specific BARF1 oncogene. For a total of 78 out of 85 NP brushings samples (92%) from NPC patients, RNA profiling could be performed using NASBA and RT-PCR. As a control for RNA quality and in order to detect general EBV transcriptional activity, we performed a NASBA assay for EBNA1 mRNA, which is expressed in all EBV-associated malignancies, including NPC.12 Sixty-seven out 78 (86%) were EBNA1 mRNA-positive, reflecting EBV transcriptional activity in NP brushing samples (Fig. 4). This was confirmed by parallel RT-PCR positivity for the noncoding BARTs, which were detected in 69% of samples tested and in 76% of EBNA1 mRNA positive samples (Table 2).

To confirm that the elevated EBV DNA loads in the NP brushings indeed reflect carcinoma-associated EBV activity, we investigated the expression of BARF1 mRNA, a carcinoma-specific viral transcript. BARF1 mRNA positivity was seen in 58/78 (74%) of all samples and in 57/67 (85%) of EBNA1 mRNA-positive samples. This carcinoma-specific EBV transcription in NP brushings directly reflects oncogenic viral activity at the anatomical site of tumour development.

In addition to BARF1 expression, we studied expression of LMP2 mRNA, which is consistently expressed in NPC tissue biopsies.39 LMP2 mRNA was detectable in 67/78 (86%) of NP brushings and in 66/67 (99%) of EBNA1-positive samples.

The first batch of NP brushing specimens analysed for EBV mRNA arrived at ambient temperature at the Amsterdam laboratory (see Materials and Methods), which may influence RNA quality. Therefore, we also separately analysed the first and second (frozen) batch of samples. For the first batch EBNA1 and BARF1 mRNA positivity was respectively 39/46 (85%) and 30/46 (65%) in brushing samples from NPC patients. The second batch (n=32) showed significantly higher mRNA positivity (28/32 (88%) for EBNA1 and 28/32 (88%) for BARF1 mRNA in NPC patients, indicating an effect of shipment conditions on RNA quality.

Remarkably, the 8 NP brushing specimens from NPC patients that were below COV in EBV DNA load assay (see Table I; EBVDNA load range 108910 EBV DNA copies/NP brushing), were also negative for EBNA1, BARF1, BARTs and LMP2 RNA, probably reflecting incorrect

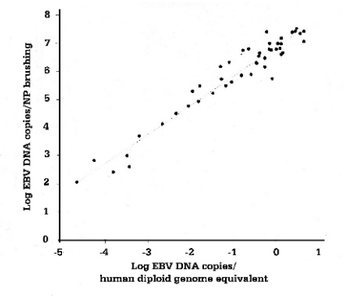

Fig 3. Linear correlation between numbers of EBV DNA copies expressed per nasopharyngeal brushing sample ("EBV DNA load/ NP brusing") or per human diploid genome equivalent

sampling. β-globin DNA copies were within the normal range for these samples.

In the control population, no EBV mRNA was detectable in the tested NP brushings, despite positivity for a low copy cellular U1A snRNP housekeeping RNA and the presence of EBV DNA This confirms our previous findings that BARF1 mRNA is a carcinoma- specific marker (10, 12, 15).

DISCUSSION

Diagnosis of primary NPC currently requires pathological assessment of a biopsy taken from the postnasal space. This is an invasive and painful procedure that may lead to extensive bleeding and cannot be repeated easily without compromising the patient. In this study, we show that detection of

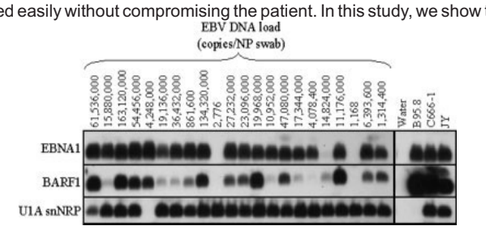

Fig. 4. EBNA1 and BARF1 RNA expression in NP brushings samples as determined by NASBA, in relation to EBV DNA load. Shown is an autoradiogram of NASBA products hybridised with a radioactive labelled internal oligonucleotide probe.

EBV DNA load and carcinoma- specific BARF1 mRNA in NP brushings provides a highly specific and noninvasive diagnostic tool for NPC. Of the 79 individuals who were above COV in the quantitative EBV PCR assay, 77 were subsequently diagnosed with NPC by histological examination of an NP biopsy and demonstration of EBV involvement by in situ EBER RNA expression. Although detection of molecular markers may not entirely replace the biopsy, as previously indicated (40, 41). EBV DNA quantification in NP brushings combined with qualitative BARF1 and EBNA1 mRNA detection may greatly reduce the number of invasive NP biopsies required. In addition, this method may be used repeatedly for post therapy monitoring and detection of local recurrent disease (study in progress). The feasibility of this approach was recently shown

(27, 42) and our ongoing study confirms the observations that high post therapy EBV DNA loads in brushing samples correlate with unfavourable clinical outcome (data not shown). Our preliminary data furthermore indicate that follow-up NP brushing sampling is a noninvasive and well tolerated prognostic strategy, without any noticeable side effects and suited for monitoring of kinetic changes in EBV DNA load during chemo- and radiotherapy (M.A. et al, in preparation).

The EBV DNA load approach has high positive and negative predictive value (Table I). It can assist in clinical patient management and it can be repeated more easily and frequently than the biopsy. NP brushing with direct storage of samples in the described DNA and RNA-stabilizing buffer (33, 34) is a relatively cheap method that, combined with a portable nasendoscope, may be used "in the field" in regions with high NPC incidence, e.g., in developing countries such as Indonesia, where medical facilities are poor. Nasendoscopy may even not be required for experienced examiners. Thus, the strategy described here may improve future population-based screening studies for confirmation of NPC presence, for example following initial risk assessment by serological screening for EBV-specific IgA and IgG (11). Monitoring of EBV parameters reflecting increased viral burden and carcinoma-specific transcriptional activity is more sensitive and specific than previously described cytological evaluation of NP brushing smears (Refs. 43,44; Harijadi et al., unpublished data).

A small number of NP brushing samples was below CoV in LC-PCR. These specimens were also negative for EBNA1 and BARF1 transcription, despite high total cell numbers in the sample as determined by quantitative human â-globin PCR. This may be because of erroneous brushing of an anatomical site with no NPC involvement or limited release of NPC cells during brushing. NPC cells at the surface may also be obscured by blood or tumour detritus (43). However, in our study, we did not find any correlation between NP bleeding and EBV DNA levels (data not shown). Moreover, there is a possibility that a deeply located tumour is missed by the NP brushing procedure, while the deep biopsy may be able to yield sufficient number of tumour cells in such case (27, 43). It is not uncommon that multiple biopsies are needed in some patients to make definite NPC diagnosis (25). The NP brushing procedure has a false negativity rate comparable to that of the diagnostic NP biopsy. Repeated brushing, however, is feasible because of its noninvasive nature compared to NP biopsies. Whether repeated brushing could give additional primary

Table 1. Specificity, Sensitivity, Positive Predictive Value (PPV) and Negative Predicitive Value (NPV) of EBV DNA load Quantification in Nasopharyngeal Brushings at different Cut-Off

	COV = Mean EBV load in controls + 2 standard deviations (1666 EBV DNA copies/brushing)		COV = Mean EBV load in controls + 3 standard deviations (=2312 EBV DNA copies/brushing)	
	Below COV	Above COV	Below COV	Above COV
NPC patients (n=85)	8	77	8	77
Controls (n = 88)	85	3	86	2
Specificity	97%		98%	
Sensitivity	91%		91%	
PPV	96%		97%	
NPV	91%		91%	

diagnostic value in individuals below EBV DNA load COV could not be assessed in our current retrospective EBV DNA load analysis, but the feasibility of repeating the brushing procedure without significantly compromising the patient is apparent from the ongoing prognostic follow-up study discussed above.

Some studies showed complete absence of EBV DNA in NP brushings obtained from healthy EBV-carriers (24, 26). This may be because of insensitive, qualitative (multiplex) PCR

assays (24) or insufficient brushing of the NP epithelium in these controls. In contrast, several other studies (25, 45) and our present study using sensitive quantitative real-time PCR, have found EBV DNA in brushing specimens from most healthy carriers, albeit at low levels. This is not surprising, as EBV-infected B-lymphocytes have a homing preference for the NP region (Waldeyer's ring) and virus is shed into the oropharyngeal space (46, 47).

It is yet unclear whether the carcinoma-specific BARF1 mRNA is consistently expressed in all NPC cases. Decaussin et al. (48) showed BARF1 mRNA expression in 85% (23/27) of EBV-positive, North African NPC biopsies (all confirmed by protein detection). In our current larger study, we found a similar positivity rate, with BARF1 mRNA in 88% of EBNA1 mRNA-positive samples. This may indicate that BARF1 expression is heterogeneous between tumours, although low RNA expression levels in a minority of tumour cells cannot be excluded. We could detect viral LMP2 mRNA in nearly all tested samples and recent studies showed that this gene may contribute to carcinogenesis (49, 50). We believe BARF1 mRNA is a better NPC marker, because LMP2 may be detectable in latently EBV-infected B-cells in the circulation of healthy EBV-seropositive individuals and is therefore not carcinoma-specific (46). However, since LMP2 mRNA is absent from control brushings, the high transcription level in tumour cells in NPC patients may be used as carcinoma marker, e.g. by using a quantitative NASBA or RT-PCR assay.

In addition to viral RNA profiling, the NP brushing specimen could also be useful for investigating EBV gene polymorphisms, mutations in human oncogenes, epigenetic changes and for cellular gene expression profiling to predict tumour behaviour and prognostication. Sensitivity and specificity of the NP brushing procedure may be increased by using a combination of molecular carcinoma markers (25, 40, 41). Cellular genetic markers, e.g. hypermethylation of numerous

Table 2. EBV RNA Positivity in Nasopharyngeal Brushing Sample Obtained from NPC Patients

EBV RNA target	all brushing samples		Brushing samples stored in -80°C	
	No. positive NP brushings/ no. tested	No. positive NP brushings/ no. EBNA 1-positive NP brushings	No. positive NP brushings/ no. tested	No. positive NP brushings/ no. EBNA 1-positive NP brushings
EBNA1	67/78 (86%)	na	28/32 (88%)	na
BARF1	58/78 (74%)	57/67 (85%)	23/32 (88%)	27/28 (96%)
BARTs	54/78 (69%)	51/67 (76%)	23/32 (72%)	20/28 (72%)
LMP2	67/78 (86%)	66/67 (99%)	29/32 (91%)	28/28 (100%)

EBNA1, Epstein-Barr nuclear antigen 1; BARF1 BamH1=A rightward frame 1, BARTs BarnH1-A rightward transcripts and LMP2, latent membrane protein 2. Data are shown for all collected brushing samples from which RNA analysed could be completed (n = 78 including, samples stored at room temperature) and from the batch of fresh samples, which was consistently stored at -80°C until RNA isolation and amplication procedures (n = 32)

tumour suppressor gene promotor regions, have been described (25, 40, 41) and could putatively be added to future multianalyte NPC screenings approaches, for example to confirm first-round serological EBV screening. This is, however, the first description of an EBV-encoded oncogene, i.e. BARF1, as a carcinoma marker in NP brushings.

Although EBV DNA load monitoring in blood, plasma or serum of NPC patients has widely been described, circulating viral DNA levels are relatively low (1002,000 copies/ml plasma or blood) in a significant subset of NPC patients thus making accurate quantification and definite diagnosis difficult (22). The circulating EBV DNA is fragmented and is probably derived from apoptosed NPC cells releasing their DNA content into the blood (22,23). Furthermore, blood samples from NPC patients are BARF1 mRNA-negative (22) indicating absence of circulating tumour cells. Thus demonstration of an elevated viral DNA burden plus carcinoma-specific viral

mRNA expression in NP brushings is preferable in patients suspected for NPC.

In conclusion, quantitative monitoring of EBV DNA and simultaneous EBNA1 and BARF1 mRNA detection in NP brushing samples is a specific, noninvasive tool for diagnosis in patients suspected for NPC, directly detecting aberrant and carcinoma-specific EBV activity at the anatomical site of primary tumour development. Because of its noninvasive nature the NP brushing method would be a valuable tool that can be used frequently during prognostic follow-up. Moreover, the NP-brush method may be particularly useful as confirmatory test for NPC risk assessment in population screening studies, using for example IgA and IgG serology to specific EBV proteins as initial diagnostic marker (11). Our approach may further support implementation of population-based screening programs in South-East Asia and North Africa, where NPC incidence is the highest. The feasibility of such an approach is underlined by studies from our institute indicating detection of human papillomavirus DNA in cervical brushings as a sensitive tool for population-based cervical carcinoma screenings programs (51, 52)

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CHAPTER 8

General Discussion

EBV infection in humans is ubiquitous; once infected, the virus persists in the host for life. Primary infection usually occurs early in life, causing little or no disease. The virus must infect B cells in order to persist in the host, as illustrated by the absence of EBV infections in children with a B cell deficiency (32). After B-cell-driven viral replication (lytic infection), new virions may spread by infecting other cell types, such as epithelial cells, monocytes, and occasionally T/NK-cells. In most cell types, lytic infection leads to cell death and the release of viral antigens. EBV virions may "quietly" spread to adjacent cells and antigens may be digested, triggering a humoral immune responses to lytic stage antigens in the host. In B-cells, the virus persists in a latent state with restricted EBV gene expression and thus evades immune elimination. B-cells carrying latent EBV can activate and replicate in a controlled fashion through a virus-imposed germinal center-like reaction. Activation and proliferation of latently infected B-cells is counteracted by an effective T-cell response that targets selective latent and (immediate) early lytic EBV antigens, thus creating a well-balanced equilibrium that ensures the lifelong persistence of EBV. When immune system is suppressed, i.e. by immunosuppressive drugs or HIV infection, EBV positive B-cells may grow-out and cause reversible lymphoproliferative disorders which may turn into aggressive malignant lymphoma, if left untreated. In the immunocompetent host, non-viral molecular abnormalities imposed by the host or the environment, such as (epi)genetic changes, chemical pollution including agents that can activate EBV (reviewed in (22, 123)), increase the chances that tumors will develop. Frequently, the malignant state is preceded by aberrant viral lytic replication, as indicated by increased antibody titers to lytic antigens at diagnosis. This implies that tumor progression provokes stronger antibody responses to lytic EBV components as a consequence of viral multiplication in the patient. Restricted EBV lytic expansion is possible because of the virus ability to overcome local immune surveillance due to defined viral different immune evasion functions operating during the lytic cycle (reviewed in Chapter 1 and (45, 46, 135)). Key viral functions contribute to impose survival and replication on genetically damaged cells, creating a basis for malignant growth (49, 50). EBV associated malignancies include several types of lymphoma and carcinoma; of these, NPC is highlighted in this thesis.

EBV-positive NPC (especially WHO types II and III) shows more aggressive clinical behavior with faster tumor growth and more metastasis compared with non-infected NPC tumors (126). Several studies have shown the consistent presence of EBV in NPC from high and low endemic areas (57). The clonality of the EBV genome (101), with expression of virus-encoded latency type II markers in the tumor cells and characteristic antibody responses to EBV, underline this association, indicating a direct role of the virus in NPC pathogenesis (41, 47). Consequently, EBV-specific components (DNA, RNA, and protein antigens) may act as tumor biomarkers that can be used to the evaluate risk for and presence of the tumor, disease burden, and response to therapy.

Because symptoms of early-stage NPC are non-specific, NPC is rarely brought to medical attention before it has spread to regional lymph nodes. Biopsy is the clinical gold standard for NPC diagnosis (127). This method is invasive and requires specialized ENT techniques. The late clinical presentation reduces the chances for effective therapy and a complete cure. At later stages, treatment is more complicated and there is an increased tendency for the disease to relapse or persist. Because most (>85%) patients present at late stages in Indonesia, there is a clear need to improve early detection of NPC. In addition, few specific NPC-diagnostic tests exist, as detailed elsewhere in this thesis. Overall, this situation requires the development of new approaches to diagnose NPC especially aimed at identifying early-stage NPC at first presentation. This preferably should be done in high-risk populations to allow early intervention. Improved medical awareness of early-stage symptoms and the availability of reliable early detection tools are

desirable.

The development of EBV-based diagnostic assays for primary diagnosis and screening of NPC are described in this thesis. The assays developed are based on anti-EBV antibody responses and molecular EBV DNA/RNA approaches. This is the first study to explore the design and use of new diagnostic methods for NPC detection in Indonesia. The study delivers novel testing methods which are simple and reliable, yet sensitive and specific, providing high positive and negative predictive values. The application of these new EBV-based approaches to individuals at "high-risk" for NPC is proposed. In the future, the use of standardized EBV-based techniques coupled with a "well-educated" society and improved environmental quality may reduce the number of patients who present with late stage NPC.

1. General diagnostic aspects

Generally, low (or undetectable) levels of EBV DNA, mRNA, and IgG/ IgA response to EBV proteins are found in healthy seropositive individuals. The opposite situation is found among NPC patients, where elevated (detectable) levels of EBV-DNA and abnormal EBV gene expression are found at the tumor site which are partially reflected in the blood circulation as well as and are preceded or paralleled by an increased anti-EBV IgG/ IgA response (53). The increase in IgG/IgA antibody responses is directed towards more diverse EBV proteins, with elevated IgA to EBV proteins characterizing NPC and reflecting the disease's mucosal origin. Nevertheless, no direct correlation between molecular markers and immune responses are observed because a rise in EBV activity does not always evoke a parallel level of antibody responses. Still, each protein individually may trigger local or systemic immune responses that reflect aberrant viral behavior associated with the development of NPC. Differences observed for these parameters can be used to disseminate healthy virus carriers from diseased ones and are advantageous for the development of diagnostic tests.

1.1. Serological approaches

NPC originates from the nasopharyngeal mucosal epithelium and characterized by infiltrating lymphocytes that secrete cytokines, such as TGF-β, IL-5, and IL-6 (52). These cytokines induce class switching and elevate IgA antibody production (83). On the other hand, elevated IgG responses reflect reactivation of B cell memory in the periphery, probably triggered by the release of viral components from the tumor site. Both aspects of humoral immunity and observed in NPC patients. EBV-related malignancies, such as NPC, occurring in immune competent individuals are capable of evoking cellular immune responses to the virus (72). Yet, the tumor escapes immune elimination through mechanisms that require further exploration, but include resistance to apoptosis and local T-cell silencing (64, 95, 97, 123). On the other hand, the viral activation that underlies NPC development may trigger a humoral immune response, but tumor-associated membrane proteins, such as LMP1 (88), LMP2 (34, 65), and BARF1 (122) fail to induce a significant humoral immune response. However, the nuclear protein EBNA1 induces a strong antibody response (reviewed in (92)) reflecting its release from tumor cells. Interestingly, antibodies to lytic viral components like EA, VCA, and MA are also associated with NPC development (see Chapter 2 and (73)) which suggests that lytic virus (re)activation accompanies the process of tumor development (49, 50). To date, however, a direct demonstration of this lytic process has not been achieved at the level of the tumor, except in the study by Zhang et al. (143) which showed very limited viral replication in only some keratinizing tumor cells of NPC. As consequence, these humoral immune responses to EBNA1 and some lytic viral antigens can be utilized to diagnose NPC, as detailed in this section and in chapters 2-5.

Multiple studies have addressed the use of individual (24, 36) EBV antigens for NPC diagnosis. This is extended in the present study by the discovery of two peptide-based markers with high diagnostic performance that allowed the development of more standardized tests. One is derived from the viral capsid protein p18 (BFRF3) and the other from the EBNA1 protein (BKRF1). In particular, in developing countries, there is a need to develop simple, affordable, sensitive, and specific diagnostic tools. The availability of synthetic peptides may allow the development of simple tests such as dipstick or filter tests that can be used in regional labs, allowing improved screening options in a standardized large-scale test format.

Serological tools that are developed in this thesis are an improvement of existing methods, yet are unable to completely cover all patients. Other studies indicated the usefulness of different types of lytic antigens, but we did not find added diagnostic value for these markers in our EBV serology system. Further work is needed to complete the serological coverage.

Old and colleagues (96) were the first to demonstrate a serological relationship between EBV and NPC using immunodiffusion antibody testing. This method was subsequently confirmed with the use of IFA (42). Up till now, IFA is still used as the gold standard for EBV and NPC serodiagnosis. IFA-staining uses glass-immobilized, intact EBV-positive cells expressing different sets of viral antigens to determine the antibody response to EBV complexes, such as EBNA, EA(D) and VCA. This method is limited by technical difficulties and by subjective interpretation. Nevertheless, this technique provided important information about the dynamics of antibody responses during primary EBV infections, asymptomatic carriers, and in diseased carriers (43). Later on, EBV immunoblotting and ELISA (Chapters 2 and 4, and (7, 99)) analysis confirmed the IFA results to a molecular detail. IFA detection of IgA responses to EA(D) and VCA complexes have been widely used in NPC diagnostic work-up (29, 41, 48). IgA to VCA shows high sensitivity (up to 97%)(127), while IgA to EA(D) displays high specificity (29, 58, 127). Therefore, IgA-VCA is used largely as a tool for primary diagnosis (19) and screening (12, 53, 94) of NPC, with IgA-EA(D) used for confirmation. Both IgA-VCA and IgA- EA(D) have also been proposed as a marker to monitor treatment (27) and predict disease relapse (107).

EA and VCA consist of a complex mixture of antigens, and the spectrum of antibody responses to each antigen varies among individuals (Chapter 2). In some studies, IFA was less sensitive compared with ELISA when using a single EBV antigen (23, 86, 108). IFA-based IgA VCA or EA failed to reliably detect the restricted antibody response that occurs in juvenile NPC patients (27, 58). Single EBV antigen ELISA formats using immunodominant markers for detection of IgA-VCA or IgA-EA may provide adequate alternatives (13, 21, 25, 115). The application of single rather than complex EBV antigens is preferred but to create diagnostic tests based on single rather than complex EBV antigens, it is necessary to consider the molecular diversity of the underlying antibody responses.

We are the first to show the molecular diversity of IgG and IgA responses to EBV antigens in NPC (**Chapter 2**). A restricted IgG response to EBNA1, VCA-p18, and occasionally to ZEBRA and VCA-p40, is observed in Indonesian individuals and confirms results observed in Caucasians (130). Healthy EBV carriers are further characterized by the absence or sporadic presence of weak IgA responses to EBNA1 and VCA-p18. In contrast, NPC patients show IgG and IgA recognition to a broader spectrum of EBV lytic antigens, such as VCA-p160, EA-p138, major EA(D) complex, thymidine kinase (TK), and DNAse. This difference between healthy EBV carriers and NPC patients is consistently observed in populations from high, medium, and low NPC endemic areas. Juvenile NPC, on the other hand, exhibits restricted IgG and IgA responses compared with adult NPC (58). Overall, the results suggest that healthy subjects worldwide are able to minimize EBV infection and spread as characterized by the limited diversity of their anti-EBV antibody responses,

whereas in NPC patients increased virus reactivation (by genetic or environmental factors) and the virus's ability to evade the immune system allows it to spread and drive tumor formation.

In our study (**Chapter 2**), the differences in IgG and IgA response-patterns to individual EBV antigens suggest dissimilar epitope selection as a consequence of differences in antigen processing and presentation. The higher diversity of IgG responses to denatured EBV antigens on immunoblots compared with IgA suggests that IgG responds more to linear epitopes, while IgA is directed more towards conformational epitopes. Although both elevated EBV-specific IgG and IgA levels, such as those in NPC, have been exploited to develop NPC diagnostic tools (30, 36, 58), IgG responses are also found among EBV-related lymphomas and in some normal healthy EBV carriers in NPC endemic areas (58, 109). Therefore IgA is preferred over IgG to develop as an NPC marker (41, 47), in keeping with the carcinoma's origin in the mucosal surface (see also **Chapter 3**).

The diversity observed for IgG-EBV immunoblots may be used to define markers for NPC diagnosis to predict relapse (Chapter 2 and (58, 120)), as well as to find marker(s) suitable for post-therapy monitoring. However, like IFA, the use of immunoblot is limited by cost (cell culture facility and cell extraction) and the expertise needed to interpret the results (107). Nevertheless, this method provides detailed information about antigen(s) that could be developed into an ELISA format. Recently, a commercial version of an immunoblot performed promisingly for NPC diagnosis (100).

Several immunoassay techniques have been developed to replace the cumbersome IFA. Recently, a microparticle bead assay (xMAP), where individual beads are coated with different EBV antigens, was proposed as a replacement for EBV-antigen expressing immobilized cells on glass slides. This new method has readings that are equivalent to IFA (2, 35), and can accommodate multiple antigens with a small serum sample volume (8, 35, 61, 81). However, this new technological assay requires a bead-sorter or hemacytometer counter (2, 35), and is relatively expensive. ELISA, on the other hand, offers a simple, sensitive, easily-interpreted semi- to fully-quantitative result, and can be used in a high-through-put format.

In continuation of the immunoblot study (**Chapter 2**), we explored EBV EBNA1 and individual lytic antigens separately in various formats to IgA-EBV ELISA. Our work on single EA and VCA-derived antigens gave unsatisfactory outcome (unpubl. data and Chapter 4). Results obtained with some purified components were in conflict with those of other studies. Overall, no consensus exists on the use of a single protein marker to diagnose NPC. This underlines the variation in antibody reactivity to multiple EBV antigens (24-26), differences in antigenic sources, selective epitope use, and population studies, making attempts to use one-type ELISA universally difficult.

Studies exploring the EBV lytic components of individual early (EA) and late (VCA) proteins have been done, with various results. ZEBRA protein is a switch between latent and lytic EBV phases; therefore, antibodies against ZEBRA should signal the activation of the EBV lytic phase. A high prevalence of IgG-ZEBRA was observed among NPC patients (especially in juvenile NPC patients), even those who were negative for IgA to VCA and EA (25, 86). Dardari et al. (26) used a peptide epitope specific for IgG-ZEBRA aimed at the detection of NPC, while others (26, 124) identified ZEBRA epitopes recognized by IgG in HIV-infected individuals and IM subjects. IgG-ZEBRA was also suggested for use in NPC prognosis to predict survival, relapse, and lymph node-involvement (26, 140). IgG reactivity to full length ZEBRA was found to be specific for IM (85%), early HIV infected, and BL subjects (50%)(54). On the other hand, IgA-ZEBRA was rarely evaluated as an additional marker for NPC (13). These studies reflect the diversity of results obtained with a simple EBV marker that does not contribute towards achieving a consensus. Other

examples have been discussed in experimental chapters (see Chapters 2, 3, and 5).

Previous studies (73, 74, 99) and our own results suggest that IgA has a better reactivity to complex mixtures of Major-EA(D) than to single EA(D) antigens, since each EA component contributes only partially to the EA complex reactivity (work of D.K. Paramita at our institute).

EBV envelope proteins play a major role in viral infection because they bind to the host cell to initiate infection. Therefore, antibodies against these envelope proteins are considered important for neutralizing viral infection. Johannsen et al. (55) were able to identify 12 different EBV glycoproteins. However, compared with EA proteins, only a few of the late EBV antigens showed diagnostic potential, i.e. VCA-p160 (80), gp125, and gp350/ 220 (136). Elevated IgA response to gp350 was observed in NPC, and lower in C-EBV and HD (137), thus being not completely specific for NPC. Recently, xMAP (luminex multi-analyte profiling) technology showed the potential use of IgA/IgG antibodies to gp78 compared with gp350/220 and BLRF1 (35). In our hands, we found only limited diagnostic value for recombinant VCA-p160 (purified from baculovirus-expression system; Middeldorp unpublished procedure) and peptides derived from it (89), as well as purified gp125 (Chapter 4). However, we and others (31, 44) found diagnostic potential for p18 antigen, a small capsid protein.

Specific EBV antigen(s) for ELISA may be generated from native antigens extracted from EBV-positive B cell lines, from purified recombinant antigens, or from synthetic peptides. Recombinant antigens require a proper expression system and excellent purification methods to avoid contamination with non-EBV (bacterial or yeast) proteins, and should enable protein to stably maintain its natural conformation. Synthetic peptides, especially when comprising a stable span of selected immunodominant epitopes, are relatively cheap compared with natural or recombinant antigen products, and are a promising antigen alternative.

PEPSCAN technology (90), using overlapping amino acids spanning the complete sequence of a specific EBV protein, is able to identify diagnostically relevant linear immunodominant epitope(s), even in a complex mixture of human serum (90). PEPSCAN defined-multiple-small-immunodominant epitopes can be combined into long synthetic peptides as shown for EBNA1 (89) or VCA-p18 (131). The EBNA1 and VCA-p18 synthetic peptides can be mixed in one ELISA well, and used to diagnose NPC (Chapter 3). Similar sensitivity and specificity were found when sera from Indonesia or a Chinese panel from Hong Kong was tested, suggesting that the system works for patients from different ethnic backgrounds. In a Tunesian NPC study, IgA-VCA-p18 was more suitable for diagnosing NPC compared with IgA-EBNA1, and was proposed to be able to predict tumor relapse (58).

Our study shows that peptide mixtures derived from EBNA1 and VCA-p18 can replace the complex mixture of recombinant EBV proteins (19, 24, 25). The large 60-amino acid peptides maintain some conformational epitopes. We found that these peptide-derived EBV antigens are recognized by the majority of the NPC populations tested. In addition, the VCA-p18 peptide was shown to be a versatile antigen, since it could be used to diagnose different EBV-related diseases, and is used for detection of IgM antibody for mononucleosis, and IgG antibody to determine EBV carriership (131).

Despite its analytical options, PEPSCAN analysis can not entirely mimic the conformational epitopes contained in full-length (complex) EBV proteins. The sensitivity of fully peptide-based IgA/[EBNA1+VCA-p18]-ELISA (here named IgA- combi ELISA) was determined to be 90% when tested in an Indonesian NPC panel, leaving 10% of NPC cases undetected. PEPSCAN analysis of VCA-p40 (BdRF1), which proved a promising antigen in our immunoblot analysis, failed to identify immundominant epitopes of linear sequence, indicating conformational-epitope involvement. All NPC cases with a negative IgA-combi ELISA result recognized VCA-p40

and VCA-p18 in the EBV immunoblot-strip. Therefore, a full length VCA-p40 gene was fused with the VCA-p18 (BFRF3) immunodominant epitopes (131) and expressed in E. coli (Chapter 4). The recombinant VCAp40+18 was purified and used as single protein in ELISA (here named IgA- VCA ELISA). Table 1 shows the result of IgA-combi ELISA versus IgA- VCA ELISA tested in parallel with NPC sera (similar data are shown in **Chapter 4**; Fig. 8).

Selected NPC samples (n=42) from our large NPC panel (n=562) including 20 positive (47.62%) and 22 negative (52.38%) samples in our routine IgA-combi ELISA IgA-combi ELISA were tested with IgA-VCA ELISA. Overall, Table 1 shows that IgA-VCA ELISA improves the detection rate from 20/42 (47.62%) to 33/42 (78.57%). When adding these results to all NPC samples collected in our study, the IgA-VCA ELISA increased the sensitivity from 90% to 96.26%. Thus, the use of EBNA1 peptide mixed with recombinant VCA-p40+18 in an IgA-ELISA should be considered. However, the eight (8) NPC samples that were negative for both IgA-ELISAs should be further analyzed using alternative EBV markers.

Currently, no single EBV antigen can be used as a sole marker in an ELISA to diagnose NPC. In our attempts, we still missed 3.74% of NPC patients using three different EBV markers. When we tested IgA-combi ELISA with a limited number of samples from Caucasians with acute IM and chronic EBV (C-EBV), the assay was negative for IM and 30% were positive for C-EBV samples. However, these diseases are infrequent in NPC endemic areas. Therefore, serological approach to measure elevated IgA reactivity to EBV antigens as developed in this thesis may be used as a NPC risk sign in the general Indonesian population and should be useful for screening purposes (53).

Table 1. Parallel test of IgA-EBV combi ELISA and IgA-EBV VCA ELISA in NPC sera (n=42)

		IgA-EBV combi-ELISA	
		+	**-**
IgA-EBV VCA-ELISA	**+**	19	14
	-	1	8

1.2. Measuring EBV DNA load and mRNA expression

Leon et al. (66) showed that higher levels of DNA are found in the serum of cancer patients compared with those who have non-malignant diseases. Stroun et al. (121) showed that circulating DNA of some cancer patients contain certain neoplastic characteristics. Numerous studies since then confirm that many neoplastic diseases can be identified by circulating DNA (11, 17, 98, 110, 114).

Elevated EBV-DNA was observed in the plasma/serum (14, 57, 75, 93, 104), whole blood (67, 103, 116), PBMC (39, 132), and tumor tissues (104, 112) of patients with EBV related diseases and malignancies (14, 16, 38, 62, 119), but was almost undetectable in seropositive healthy individuals (6, 16, 78, 139). Real-time PCR is the latest technology available to measure EBV viral load. It is based on the amplification of a short conserved DNA sequence using either an intercalating dye or a fluorescent probe to quantify the products against a series of standards of known EBV DNA content (104, 116). This method eliminates product carryover and post amplification detection, and offers a more sensitive detection method than gel electrophoresis. DNA quantification had greater flexibility when applied in different target panels (14). The quantification of EBV-DNA can utilize the amplification of unique conserved-segments within the EBV genome such as BAMH1-W, BKRF1 (EBNA1), BNRF1 (p143), BNLF1 (LMP1), BNLF2a

(LMP2A), BALF5 (Pol-1), or BZLF1 (ZEBRA) (63, 71, 78, 87, 104). Importantly, to ascertain reliable detection, the choice of a particular gene for PCR amplification should consider the (lack of) mutation frequency in that gene, which differs for certain EBV genome regions. In NPC, EBV-DNA quantification has been proposed for use in primary diagnosis, to reflect tumor burden, to monitor therapy and predict relapse (30, 69, 70, 77, 78, 82, 111, 118). Currently, there is no consensus or standard for EBV-DNA quantification, although many kits and EBV-DNA standards are commercially available (reviewed in (91)).

Development of EBV-DNA quantification methods based on amplification of a highly conserved region of the EBNA1- gene with standardized LightCycler-PCR has been discussed in detail by Stevens et al. (116, 117), and was adapted in our NPC study (Chapters 6 and 7). The EBNA1 gene segment chosen for amplification is highly conserved among EBV isolates worldwide, and proven suitable for EBV detection in samples of a variety of EBV disease syndromes, including transplant recipients and HIV carriers developing lymphoproliferative syndromes, Hodgkin and Burkitt lymphoma patients, patients with acute and chronic EBV infections, as well as NPC patients. EBNA1 is a single copy gene; therefore, one can precisely determine the EBV-copy number in a sample. Samples can be collected in GuHCN-based buffer, which provides easy, stable sample storage. The silica-based extraction removes endogenous and exogenous PCR inhibitors such heparin, hemoglobin, and lipids, as well as enabling simultaneous DNA and RNA extraction.

1.2.1. Whole Blood

The dynamic change in time of EBV-DNA load in whole blood has been used to predict the onset of PTLD, monitor therapy, and predict relapse in various types of EBV-related malignancies (reviewed in (37)). EBV-DNA quantification in blood plasma/ serum has been suggested to provide a sensitive and specific marker for diagnosing some EBV-related diseases. The presence of fragmented EBV-DNA in the plasma of patients with any of the several EBV-related malignancies (33, 116), including NPC (Chapter 6), may reflect apoptosis of EBV-infected cells rather than active viral replication, releasing random, partly digested, short EBV DNA fragments (15, 105). EBV-DNA quantification using plasma/ serum as an EBV-DNA source should include an amplification of human DNA sequences to determine whether the truncated EBV-DNA is caused by in vivo or in vitro processes. The short-sized EBV-DNA in serum may be part of the in vitro process, as coagulation may cause physical stress that may release DNA from damaged cells, thus the actual DNA copy number in serum may be overestimated (116).

Previous findings (116) showed that EBV-DNA load is mostly confined to the cellular compartment of peripheral blood. Encapsulated EBV DNA was found in the majority in whole blood specimens from PTLD patients, Burkitt's lymphoma, HIV-infected individuals, and individuals with IM (5, 102, 116, 117). The use of unfractionated whole blood has several advantages, and uses uniform, simple, standardized samples. It contains all compartments that may harbor EBV, thus reflecting the total EBV burden. Therefore, the use of whole blood to diagnose NPC was evaluated in our NPC studies (Chapter 6). In NPC patients, when positive, we found that whole blood samples contain fragmented cell-free EBV-DNA rather than encapsulated DNA, because a 99 bp amplicon provides a higher detection rate (85.9%) compared with a 213 bp amplicon (72.5%). The absence of detectable viral RNA expression in whole blood of NPC patients, confirms the absence of viable NPC tumor cells in the circulation, which is different from lymphoma patients. Apoptosis produces EBV-DNA fragments of less than 180 bp in length (15). By comparing published sensitivities of EBV-DNA load for NPC diagnosis from different studies (**Chapter 6**, Fig.2) we showed that shorter sized DNA amplicons provide higher NPC detection rates in blood samples. With a cut-off value of 2,000 DNA copies/ml blood (EBV-DNA load maximum of the normal healthy population), we found

a sensitivity of 29.5% and 60.4% for the 213 bp and 99 bp amplicons, respectively for detecting NPC in our Indonesian cohort. Therefore, we consider whole blood EBV-DNA quantification not useful for the primary diagnosis of NPC.

EBV-DNA quantification based on BamH1-W amplification is considered the most sensitive method (104, 111). This system amplifies short DNA sequences (75 bp; see **Chapter 6**, Fig. 2) isolated from high volumes of serum/ plasma. BamH1-W is a reiterated gene sequence with 7-11 repeats/EBV genome (1, 37), which varies among EBV isolates (56). Differences between EBV strains and standards may lead to incorrect estimation of the viral copy number. Recently, plasma EBV-DNA load based on BALF5 (Pol-1; a single copy gene) amplification (60) was suggested to have a higher accuracy for the primary diagnosis of NPC and in predicting treatment outcome compared with BamH1-W (63). Similar conclusions may be valid for the EBNA1-based PCR applied in this thesis.

1.2.2. In situ

Primary NPC lesions may be submucosal and easily missed by endoscopic examination, making tumors difficult to detect at early stage and causing them to remain undiagnosed until metastasis has occurred. The current gold standard is a biopsy with a pathological examination to define the cellular composition at the site of suspected malignancy, preferably with EBER staining which is the EBV diagnostic gold standard (3). However, taking a biopsy in the nasopharyngeal space (NP) is an invasive procedure and posed significant discomfort to the patient. Therefore, alternative procedures are suggested, not only for the primary diagnosis, but in particular also for repeated use to monitor local treatment effects. Exfoliative NP cytology is an alternative used for NPC patients who refuse to undergo biopsy. Application of a cotton swab to collect nasopharyngeal secretion combined with PCR detection of EBV-DNA yielded promising results (113). However, a fiber (cotton)-tipped swab can only captures superficial epithelial cells. Therefore, a nasopharyngeal brush was proposed to provide higher cell yields (84, 128, 133), and provide adequate information to determine NPC status. Brushing such as applied in cervical cancer screening, is useful to scrape/collect cells from the epithelial surface. NP brush sampling provides a simpler, less invasive method (compared with biopsy), and may be performed more frequently than a standard biopsy to monitor patients or those suspected of having NPC. This method can be applied with or without nasoendoscopy guidance (128). Standardized procedures must be used to avoid variability in the results among patients.

Unlike whole blood specimens, EBNA1-based EBV-DNA quantification of nasopharyngeal-epithelial cells has a sensitivity and specificity of 90% and 98%, respectively (Chapter 7), confirming a similar high sensitivity and specificity as that applied to NP cells in BamH1-W based DNA amplification (125). The presence of large molecular EBV-DNA (213 bp) and of EBNA1/ BARF1 mRNA shows that intact epithelial cells are the source of EBV-DNA. This demonstrates that the nasopharyngeal brush EBV-DNA assay accurately reflects local, in-situ tumor status in contrast with peripheral blood. A negative EBV-DNA load in some NPC patients (with normal copy numbers of cellular DNA, but negative for EBV-mRNA) indicates improper sampling, storage, or transport. By using NP brush specimens, we found a wide and significant difference of DNA-EBV loads between the NPC patients and control populations. Very high EBV-DNA loads (up to 10^7 cells/ brush) are found among NPC samples; while low levels of EBV-DNA load (maximally 1.5×10^3 cells/brush) are found among the normal healthy EBV carrier population. However, we did not find a correlation between EBV-DNA load and tumor stage, which may be expected because the brush only scrapes a small area of the NP surface. Because NPC brushing provides direct information, the application of this method to predict local tumor recurrence after

therapy is worth-pursuing.

In addition, epithelial-RNA isolated in parallel with DNA using the silica-based isolation technique (9) provides additional relevant information. We can detect the presence of BARF1 (and EBNA1) mRNA in NPC samples (Chapter 7) by using the NASBA technique, which allows specific RNA amplification in a DNA background. BARF1 is thought to play a role in EBV-related carcinoma, but not in other EBV-related malignancies (10, 40). Therefore, in contrast to blood with elevated EBV-DNA loads where BARF1 mRNA was consistently negative, it provides a carcinoma-specific marker and reflects the presence of viable tumor cells. BARF1 protein is barely detectable in tumor cells because it is rapidly and completely secreted (51, 106, 129). Short peptides derived from BARF1 were recently reported to evoke $CD8^+$ T cell responses (85). However the detection of antibodies against BARF1 was only reported once using a highly artificial system (122), and has not been confirmed by others. Hence, in contrast to the quantification of EBV-DNA based on EBNA1 amplification, which can be applied in many types of EBV-related diseases (116), the mere detection of BARF1 mRNA could be a promising marker for the detection of NPC directly, and deserves further evaluation.

1.3. EBV viral load in combination with serology

The dynamic fluctuation of EBV-DNA load provides direct information about active disease, while EBV serology only provides indirect evidence of an infection. It may take weeks to observe dynamic changes in the antibody responses due to the long half life of immunoglobulin compared with changes in DNA copy number of the virus, which show rapid changes that correspond to viral or tumor cell (apoptotic) activity. Considering the high sensitivity and specificity and rapid processing of EBV-DNA load measurements, it was suggested that it be used for early/ primary NPC diagnosis (111, 112), as well as to predict disease relapse (30, 76). For these reasons, we analyzed NPC samples (n=97) tested in parallel with IgA-EBV combi ELISA and EBV-DNA load determined from NP brushing, as shown in Table 2 below.

Table 2 shows that, although they address different parameters, the sensitivity of IgA-EBV and EBV-DNA load are similar (88.65% and 89.69%) indicating that both tests may be used to diagnose NPC. However, real-time PCR requires specific “clean” lab space and equipment, and is an expensive method to use for screening and daily patient-care in developing countries like Indonesia. Therefore, we propose using EBV serology as the first line NPC diagnostic test with EBV-DNA load from brushings as confirmation for the presence of a NPC tumor. Patients with negative results for both tests should be considered as not having NPC, and given an alternative diagnosis (69).

2. Proposed Application

EBV-based tests for the detection of NPC, accompanied by a standardized sampling system, may be applied for various purposes, ranging from primary diagnosis (this thesis), treatment monitoring and relapse prediction, population or high-risk group screening for early stage disease in order to achieve down-staging of the tumor at presentation.

2.1. Primary diagnosis

NPC often presents in the later stages with cervical metastasis, sometimes with a small primary or submucosal tumor that is difficult to observe. In daily practice, this may require multiple biopsies at different sites to locate the primary tumor. Elevated levels of IgA-EBV in plasma/ serum specimens and EBV-DNA viral loads in NP-brushings have shown to closely associate with NPC presence and should aid in the confirmation of NPC status in combination with CT-scans

(commonly used to define the stage of malignancy). Therefore, invasive multiple biopsies can be avoided. Cytological examination of NP cells in the brushing can also be used to determine the presence of malignant cells. However, earlier studies with samples derived from cotton swabs had limited sensitivity (113). EBV-DNA load in blood at primary diagnosis is thought to have predictive value, because low plasma EBV-DNA levels at the early stages of NPC is associated with good

Table 2. IgA-EBV combi ELISA compared with parallel EBV-DNA viral load in NP brushings in a NPC panel (n=97)

		IgA-EBV combi ELISA	
		+	**-**
EBV-DNA/brush	**+**	79	8
	-	7	3

treatment outcome (68, 76). This is not confirmed in our studies (Chapter 6) and the diagnostic/ prognostic use of EBV-DNA loads in blood or plasma should be substantiated further.

2.2. Treatment and Post-Treatment Monitoring

In Indonesia, due to its late clinical presentation, NPC tends to relapse within two years after therapy (Hariwiyanto et al., unpubl. data). The application of EBV-based assays could be extended to monitor the success of therapy and for follow-up after therapy to identify early relapse of the disease or to predict patient's survival, with the application of routine EBV-based tests parallel to therapy and clinical check-ups. Monitoring decreases in the level of EBV-DNA load (with its dynamic changes) in blood or brushings during treatment may provide information about successful therapy by showing a reduction in the biological activity of the tumor, as previously shown by Lo et al. (79) by using peripheral blood component.

During follow up after therapy, a decrease or stabilization of IgA-EBV titers accompanied by a decrease in EBV-DNA viral load may be associated with regression of the tumor and a stable physical condition. On the other hand, a rise in EBV-IgA antibody titers and or EBV-DNA load may indicate progressing or relapsing disease, as suggested by other studies (18, 30, 59, 111, 120). An ongoing study to further substantiate clinical value of serial post treatment dynamics of IgA-EBV and EBV-DNA viral load is being undertaken in our institute. We propose that in addition to the rise of IgA-EBV titer, local treatment failure may be detected by nasopharyngeal EBV-DNA load, while distant metastasis may be detected by EBV-DNA load in peripheral blood. As an additional confirmation, with its minimal side effects, nasopharyngeal brushing may be used to monitor treatment, and could replace the invasive biopsy when patients refuse to have one.

2.3. Screening for NPC

High risk individuals may benefit from the application of a tumor biomarker for screening purposes, which may lead to early detection, early clinical intervention, and a better prognosis. The screening tool(s) proposed should possess high sensitivity, and be easy and safe to use as well as cost-effective and culturally acceptable. Cost-effective screening programs should target high risk individuals; this is especially important when screening for a relatively low incidence disease, such as NPC.

In Indonesia, only a small number of NPC cases are registered in hospitals, because of

failing awareness and referral systems and a lack of country-wide registry. This allows for potential hospital bias when using single-center based incidence data. A “tip of the iceberg phenomenon” is suggested with potentially higher NPC incidence in the community, with endemic high incidence sites scattered throughout the country. Environmental exposure to (co-)carcinogenic substances--dietary and non-dietary--, as well as the genetic make-up of the population are thought to play significant roles in the different prevalence of NPC observed in different regions. With its cultural diversity, different regions in Indonesia may have different environmental exposures that are related to both dietary and non-dietary factors. Creating improved awareness of early-stage symptoms and diagnosing NPC early in its onset allows more effective clinical intervention and a better prognosis for the patient. A cost-effective screening program for NPC should be supported by effective treatment, patient management, and follow up.

Epidemiological surveys to identify NPC risk factors in endemic regions began in the late 70's (141). Symptoms related to NPC were identified, such as a series of different but general complaints in the head and neck area (53); risk factors such as familial NPC (20, 94), and certain food environmental exposures, in particular formalin and/or consumption of dried salty fish containing nitrosamines (4, 20, 134, 138) were also suggested to play a role in NPC pathogenesis. The observed clusters of NPC “hot-spots” were subjected to further exploration for potentially specific local risk factors, and later to systematic screening efforts to identify those at risk or to identify those with early onset of NPC (28, 53, 142). Many studies include IgA-EBV tests for NPC screening purposes. Individuals with elevated IgA responses to EBV have a higher relative risk (RR) for NPC diagnosis than those without or with low IgA-EBV. Ji et al. (53), in a 15 year follow-up of more than 40,000 individuals, recently suggested that elevated IgA responses to EBV may precede the clinical onset of NPC within a defined period of about two years in which NPC becomes clinically manifested. In contrast, Yang et al. (139) showed that EBV-DNA quantification in serum had no significant use in screening high-risk NPC individuals. The latter may relate to the inconsistent presence of EBV-DNA in the circulation as described in **Chapter 6.** The application of IgA/EBV screening in a cluster of individuals with known risk factors may increase the chances of identifying individuals with early stage NPC, and provide a more cost-effective screening program.

NPC screening can be performed at the clinical and community level. In the clinic, patients with recurrent head and neck complaints (53) should be considered as a high risk group for NPC screening. Such a study was recently started in our institute with promising results (Hutajulu et al., study in progress). In the community setting, screening programs should preferably target identified “hot-spot” areas, where there are potentially prevalent NPC risk factors. Simpler blood sampling methods, such as finger prick blood collection (dried blood sample/DBS) may be used instead of venous blood sampling (**Chapter 5**). Based on our work (unpubl. data), DBS is more acceptable to subjects compared with venous blood sampling, since it is less invasive, rapid, and only requires a small volume of blood sampled from the tip of the finger. DBS does not require special treatment during transport, and serum eluted from it may be used to determine IgA-EBV status. In addition, DNA samples may also be extracted from DBS for other study purposes.

During NPC screening, individuals with elevated IgA-EBV titers may be subsequently asked to undergo NP brushing in order to obtain a sample for EBV-DNA load analysis. NP brushing may be done with or without nasoendoscopy as long as standardized sampling procedures are used. Technically, this is a safe procedure with minimal discomfort for patients that can be performed with local anesthesia. Bleeding during the procedure usually indicates abnormal vascularisation of the NP site and may suggest the presence of a tumor. EBV-DNA load above CoV suggests viral activity in the NP site. Double-positive EBV IgA and DNA load results would strongly indicate presence of NPC and provide a clear sign for further clinical examinations, including a CT

scan. However, a CT scan sometime misses small pre-lesions; therefore, regular inspections (1-2 times/year) and observing the dynamics of IgA titers and/or EBV-DNA viral load is required in the final diagnosis of the NPC status.

In conclusion, in agreement with others, we found that multiple EBV markers are required to obtain a good NPC detection. As an addition, BARF1 mRNA as NPC specific marker should be further studied to define the best NPC detection system. Within this study we were able to develop new EBV-based approaches aimed for primary diagnosis and screening of NPC. When combined with easier and less invasive sampling methods, the assays developed provide relatively inexpensive, sensitive, and specific methods, particularly suited for developing countries. The application of these methods in the diagnosis and screening of NPC in the clinical setting is in progress. However, in providing appropriate and effective health care, it is important to differentiate between the application of the assays in clinical areas where patient care is the issue, and in the general public where secondary prevention programs such as risk assessment, early disease detection and intervention are the primary focus. Etiological studies to identify NPC risk factors in Indonesia should be conducted as well, to provide the basis for NPC prevention.

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